TRIMETHOPRIM-RESISTANT DIHYDROFOLATE REDUCTASE GENES IN SOUTH AFRICAN ISOLATES OF AEROBIC GRAM-NEGATIVE COMMENSAL FAECAL FLORA

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ABSTRACT

Resistance to antimicrobial agents is an increasing problem, especially in developing countries where resistant strains have undermined the effectiveness of antimicrobial agents such as trimethoprim. Since the commensal faecal flora are strongly implicated as a reservoir for resistant organisms. A survey was conducted in South Africa to determine the incidence of resistance in aerobic Gram-negative commensal faecal flora.

Faecal specimens from 272 out of 361 (75%) healthy volunteers carried trimethoprim-resistant bacteria; 357 trimethoprim-resistant strains were isolated. Trimethoprim resistance was transferable by conjugation in 55% of the isolates. The majority of the isolates were resistant to other antimicrobial agents including ampicillin 71.4% and tetracycline 88%. Most of these resistance phenotypes co-transferred with trimethoprim resistance. Analysis of 189 plasmids revealed 107 different restriction profiles which indicated that there is a large gene pool of trimethoprim-resistant plasmids in the faecal flora.

High-level resistance to trimethoprim (MIC $\geq$ 1024mg/l) occurred in 98.6% of the isolates suggesting that resistance in these isolates was mediated by the production of additional trimethoprim resistant dihydrofolate reductase (DHFR) genes. To determine the epidemiology of these genes, oligonucleotide probes were designed from the nucleotide sequence of a heterogeneous region which occurs within all trimethoprim resistant DHFR genes. Hybridisation experiments revealed that contrary to all previous data, the most prevalent DHFR of the transferable genes which hybridised was the type Ib (30%), followed by the type VIII (23%), V (13%), Ia (6%), VII (3%) and XII (0.5%). On the other hand the type VII, (38%) was the most prevalent dihydrofolate reductase gene in the 161 (45%) isolates which did not transfer their resistance factors, followed by type Ia (25%), type Ib (12%), type V (2%) and type VIII (0.5%).

In selected isolates which were unable to transfer trimethoprim resistance, the potential mechanisms of spread of resistance were investigated. The type Ib and V DHFR genes were shown to occur on non-transferable plasmids which is in stark contrast to the types Ia and VII DHFRs, the majority of which were integrated into the chromosome, and were associated with the integrase genes of Tn*7* and Tn*21* respectively. These data show that resistant DHFR genes have distinct preferences for their genetic location.

The DHFR gene from one of the isolates which did not hybridise to any of the gene probes was further studied. This gene exhibited unique biochemical properties, and subsequent cloning and sequencing of the gene revealed a novel DNA sequence which shared 85% nomology with the type XII DHFR gene. The gene was flanked by the ORF of the integrase gene of Tn*21* and appears to be inserted in a cassette-like manner. The remaining unidentified DHFR genes were probed with a specific oligonucleotide probe for this gene and were detected in 50% (45/90) of the isolates which failed to hybridise to any of the DHFR probes. This unique gene has been named *dhfrXIII* and its translation product DHFR type XIII.

DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Peter Vincent Adrian

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ABBREVIATIONS

A	adenine
Ap	ampicillin
Ak	amikacin
bp	base pairs
C	cytosine
Caz	ceftazidime
Cm	chloramphenicol
Ctx	cefotaxime
Cz	cephazolin
d	day
DHF	dihydrofolate
DHFR	dihydrofolate reductase
dhfr	dihydrofolate reductase gene
DM	Davis Mingioli minimal salts medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FH_2	dihydrofolate
FH_4	tetrahydrofolate
G	guanine
Gm	gentamicin
ID_{50}	drug concentration required to inhibit enzyme activity by 50%
In*X*	integron (where *X* is the number of the integron)
IS	insertion sequence
kb	kilobases
KDa	kilo Daltons
K_i	constant of inhibition or dissociation constant of the enzyme inhibitor
Km	kanamycin
K_m	Michaelis constant
log	logarithm
MIC	minimum inhibitory concentration
min	minutes
M_r	molecular weight (Daltons)
Mtx	methotrexate
NA	nalidixic acid
NADP+	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Net	netilmicin
OD_x	optical density (*x* indicates the wavelength in nm)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
Prl	piperacillin
Pu	Purine
r	resistant

Rif	rifampicin
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate (sodium lauryl sulphate)
Sp	spectinomycin
SSC	(0.15M NaCl; 0.015M Na_3Citrate)
Sm	streptomycin
Sx	sulphonamide
T	thymine
Tc	tetracycline
TD_{50}	time at 45°C, in minutes, giving 50% inhibition
Tn	tobramycin
Tn*X*	transposon (where *X* indicates the number of the transposon)
Tp	trimethoprim
Tris	tris(hydroxymethyl)methylamide

The standard single and three letter abbreviations are used for the amino acids.

CONTENTS

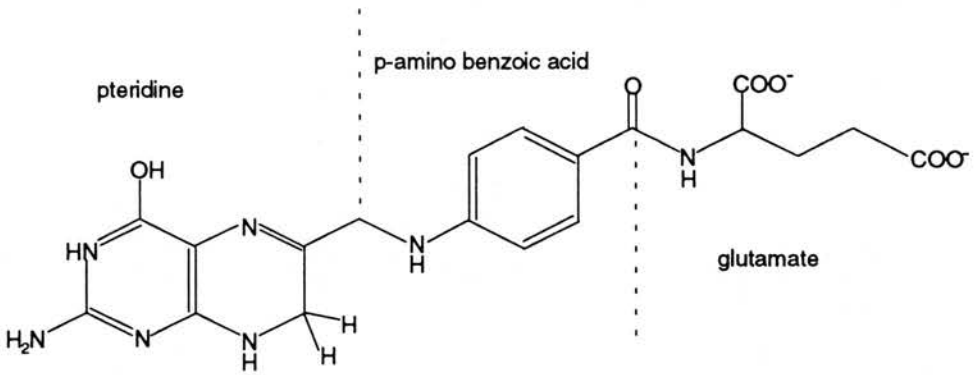
1.0 INTRODUCTION

"Everything is a matter of degree."

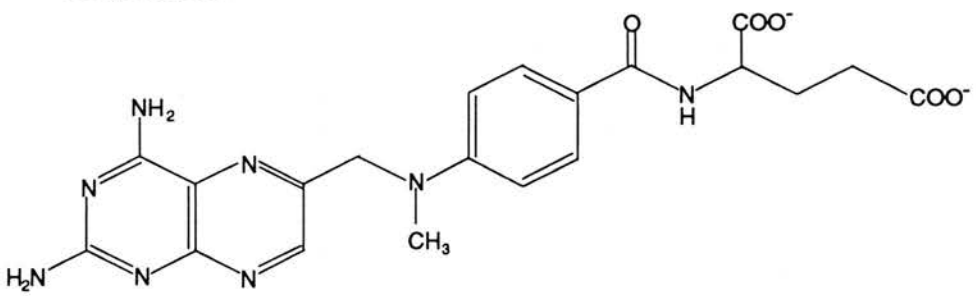
Bart Kosko "Fuzzy Thinking"

1.1 Development of diaminopyrimidines

The discovery of sulphonamides by Domagk (1935) and the subsequent elucidation of their mechanism of action by Woods (1940) led to the concept of an antimetabolite. This concept was exploited at Burroughs Wellcome who set up a laboratory in 1942 under George H. Hitchings and was a productive interaction between basic biochemistry and attempts to synthesise new antimicrobial agents (Burchall 1979). While Hitchings *et al* (1950) focused on purine and pyrimidine analogues in particular antithymines, other workers focused on folate-like inhibitors such as the close analogues of folic acid: amethopterin (methotrexate) and aminopterin (Figure 1.1).



Dihydrofolate



Methotrexate

Figure. 1.1. Structures of dihydrofolate and the analogue inhibitor, methotrexate.

It was found that the folic acid analogue 'X-methylfolate' (amethopterin) could synthetically induce the characteristics of nutritional deficiencies in animals which occur as the result of folate deprivation (Franklin *et al* 1947). Aminopterin synthesised by Seeger *et al* (1947) from Lederle Laboratories was also found to induce this deficiency state and was the first antifolate compound to undergo clinical evaluation. This work did not produce any folate-like compounds with superior activity over methotrexate. These compounds have made important contributions in the treatment of neoplasms (Burchall 1979).

Meanwhile Hitchings *et al* (1948) continued their work on small molecule antimetabolites and showed that the diaminopyrimidine derivatives such as 2,6-diaminopurine and 2,4-diamino-6,7-dimethylpteridine were antagonists of folic acid. Hitchings *et al* (1950) later published the results of a study of thymine analogues that showed that their activity was essentially antifolate rather than antithymine in *Lactobacillus casei*. These molecules produced diverse antimicrobial effects and in order to optimise their antimalarial and antibacterial properties, a large number of 5-benzyl-2,4-diaminopyrimidines was synthesised (Falco *et al* 1951; Roth *et al* 1962). This structural class was regarded as being more important since Roth *et al* (1962) noted that substitutes other than 2,4-diaminosubstituents at the pyrimidine ring were not active.

These compounds were also tested for antimalarial activity, and it turned out that for high antimalarial activity the 6-position in the pyrimidine ring had to be substituted with an alkyl (optimally methyl) group, and the benzene ring should preferably carry a halo- or nitro-substituent in the para position. This led to the development of the antimalarial agent pyrimethamine. Removal of the 6-alkyl substituent considerably depressed antimalarial activity, but increased antibacterial activity (Roth *et al* 1962).

Following the observation that for high antibacterial activity, the 2,4-diaminopyrimidines required H in the 6-position and suitable substituents in the 3,4 and 5 positions of the benzyl ring, these substituents were optimised. Figure 1.2 (Then 1993) shows the structures of some of the antimicrobial 2,4-diaminopyrimidines. It was found that 3-methoxy was superior to 3-ethoxy against *Proteus vulgaris,* and this holds for the 4-position. Higher alkoxyl groups decreased activity against *P. vulgaris*, but increased activity against *Staphylococcus aureus* (Roth *et al* 1962).

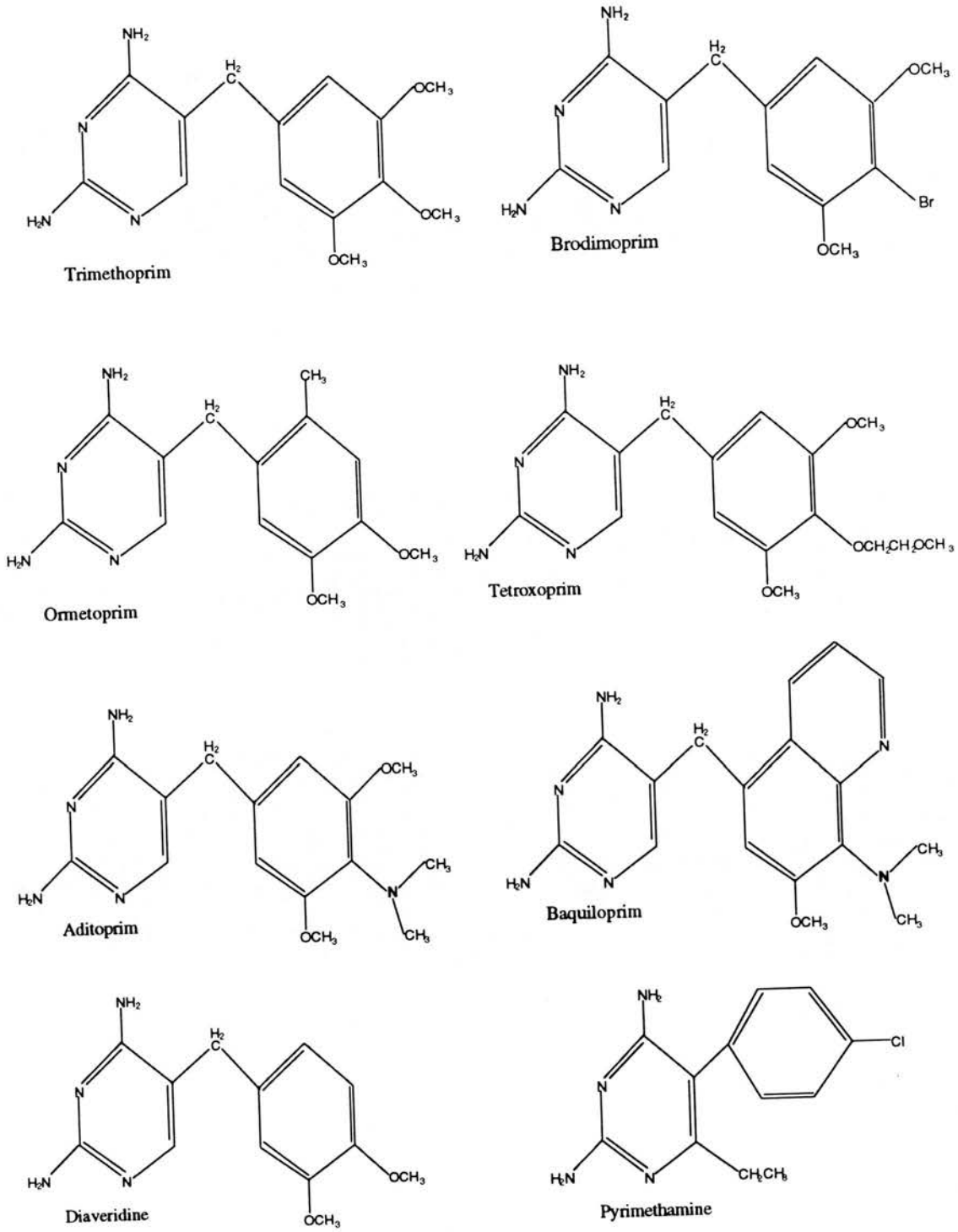


Figure 1.2. Structures of antimicrobial diaminopyrimidines.

During this work it was shown that one particular compound, the 3,4,5-trimethoxyl derivative stood out for its broad spectrum of antibacterial activity. Consequently 2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine was selected for clinical trial in 1959 (Roth *et al* 1962) and was given the name trimethoprim (BW 56-72).

Clinical trials with the early inhibitors produced malaise and nausea in some patients. In retrospect, the doses chosen were unnecessarily high (1-2g/day). However, with experiments on monkeys, it was shown that these side effects were not a property of all diaminopyrimidines, and trimethoprim was essentially free of these side effects and was selected for further development in 1959. The first clinical results with trimethoprim were presented in 1961 (Then 1993). Trimethoprim was released in the late 1960s as a combination with sulphamethoxazole called co-trimoxazole (Bushby and Hitchings 1968). Since then a number of other diaminopyrimidines have been approved for clinical and veterinary use on their own and in combination with sulphonamides as shown in table 1.1 (Then 1993). In 1993 brodimoprim was approved for clinical use, and shows a similar spectrum of activity to trimethoprim, but has improved pharmacokinetics and a longer half-life (Weidekamm 1993).

Table 1.1. Marketed DHFR-inhibitors and their sulphonamide combinations.

For human use

Combination	Use	Tradename
Trimethoprim	Antibacterial	Trimpex, Proloprim
Trimethoprim-sulfamethoxazole	Antibacterial	Bactrim, Septrin, Sultrim
Trimethoprim-sulfadiazine	Antibacterial	Nibrisin, Triglobe, Codiazine
Trimethoprim-sulfamoxole	Antibacterial	Supristol, Adisept
Trimethoprim-sulfametrol	Antibacterial	Lidaprim, Maderan
Trimethoprim-Sulfamerazine	Antibacterial	Berlocombin
Trimethoprim-sulfamethopyrazine	Antibacterial	Kelfiprim
Tetroxoprim-sulfadiazine	Antibacterial	Tibirox, Sterinor, Prim, Berco-B
Pyrimethamine	Antimalarial	Daraprim
Pyrimethamine-sulfadoxine	Antimalarial	Fansidar, Suldox, Malocide
Pyrimethamine-dapsone	Antimalarial	Maloprim
Pyrimethamine-sulfalene	Antimalarial	Metakelfin

For veterinary use

Combination	Use	Tradename
Ormetoprim-sulfadimethoxine	Coccidiostat	Rofenaid
Ormetoprim-sulfamonomethoxine	Coccidiostat	Ektecin
Diaveridine-sulfadimethoxine	Coccidiostat	Rofenon
Trimethoprim-sulfadimethoxine	Antibacterial	Trafigal
Trimethoprim-sulfadoxine	Antibacterial	Borgal
Trimethoprim-sufatroxazole	Antibacterial	Leotrox, Potrox
Trimethoprim-sulfadiazine	Antibacterial	Tribrissen
Trimethoprim-sulfachloropyridazine	Antibacterial	Cosumix
Baquiliprim-sulfadimidine	Antibacterial	Zaquilan

1.2 Mechanism of action of trimethoprim

Between 1950 to 1958 the details of the folate pathway were elucidated. Figure 1.3 shows the pathway of the synthesis of tetrahydrofolate and its roles in bacterial metabolism as reviewed by Hartman (1993).

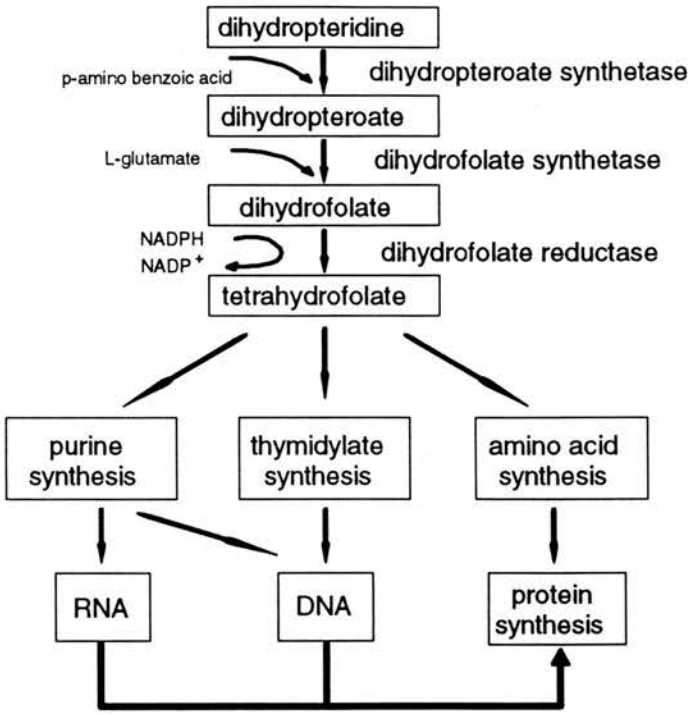


Figure 1.3. The biosynthesis of tetrahydrofolate and its role in cell metabolism.

Osborn *et al* (1958) showed that methotrexate inhibited chicken liver DHFR at known concentrations. Burchall and Hitchings (1965) published results that showed that the diaminopyrimidines also inhibited DHFR and showed that the differences in the selectivity of these compounds occurred at the molecular level and not at the pharmacokinetic level. They performed extensive studies in which they analysed the binding of various inhibitors to DHFR from different sources. It was from these studies that they realised that the diaminopyrimidines, unlike methotrexate, exhibited a high degree of selectivity for the bacterial DHFR, but not the mammalian enzyme (Table 1.2). They also noted a strong correlation between the *in vitro* activity and the strong binding of antibacterial diaminopyrimidines to *E. coli* DHFR. Then and Angehrn (1979) found that the low trimethoprim sensitivity of *Bacteroides* and *Clostridium* species was due to the production of a trimethoprim-resistant DHFR.

This lack of effect on the anaerobic bowel flora can be regarded as an advantage (Burchall 1979).

Table 1.2. Trimethoprim inhibition of DHFR from various sources.

	DHFR ID_{50} (nM)		
Source of DHFR	Trimethoprim	Pyrimethamine	Methotrexate
Escherichia coli	5	25 000	6
Proteus vulgaris	4	15 000	5
Staphylococcus aureus	5	3 000	1
Plasmodium berghei	7 000	50	70
rabbit	370 000	500	60
rat	260 000	700	90
human	300 000	1 800	90

At first sight the inhibition of the reduction of dihydrofolate (FH_2) to tetrahydrofolate (FH_4) may not appear as effective as for example the prevention of cell wall biosynthesis by β-lactam antibiotics. However the importance of the product of the reaction catalysed by DHFR, tetrahydrofolate, lies in its involvement in a whole variety of other biosynthetic reactions as a cofactor, carrier of one carbon moieties and reducing agent. Thus the biosynthesis of thymidylate, purine nucleotides, methionine, serine, glycine and several other compounds are dependent on a supply of tetrahydrofolate (reviewed by Hartman 1993). Most bacteria must synthesise folates *de novo* as shown in figure 1.3 and although a few bacteria do have uptake systems for folates, the reduction of dihydrofolate to tetrahydrofolate is a universal requirement (Hitchings 1973).

In most of the reactions tetrahydrofolate acts as a cofactor shuttling one carbon units in various oxidative states, bound to the N^5 and N^{10} atoms. In most of these reactions the tetrahydrofolate is not used up, it merely cycles from loaded to unloaded states (Hitchings 1973). If this were the only type of reaction in which tetrahydrofolate was involved, tetrahydrofolate depletion would only be possible by dilution of the tetrahydrofolate pool caused by consecutive cell divisions (Hartman 1993). This is not an effective method of preventing cell growth. However one reaction, thymidylate synthesis, involves the reduction of a single carbon moeity at the expense of the H atoms from C-6 of the pyrimidine ring of tetrahydrofolate and therefore oxidises tetrahydrofolate back to dihydrofolate (Dale and Greenberg 1972). This reaction

creates a demand for the restoration of the tetrahydrofolate pool which is a prerequisite for cell growth. Figure 1.4 shows the thymidylate cycle and the role it plays in the reduction of the tetrahydrofolate pool.

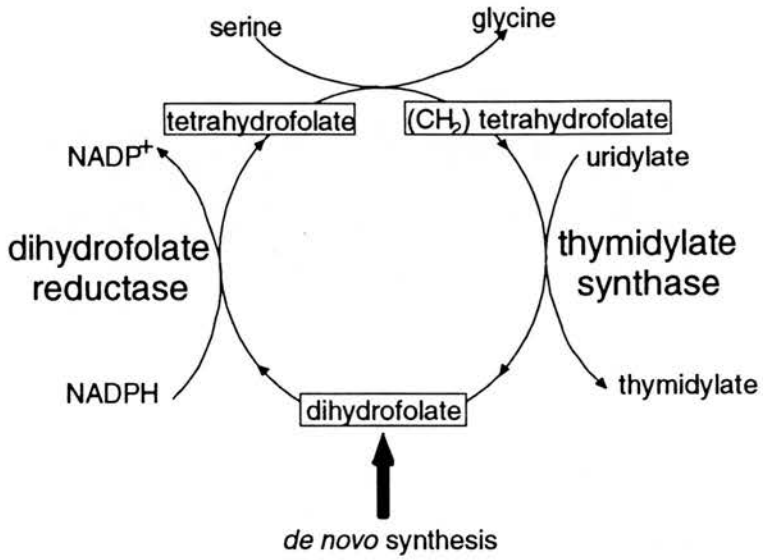


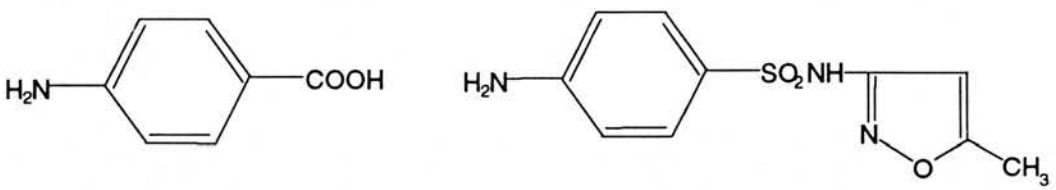
Figure 1.4. The thymidylate cycle.

The nature of the antibacterial activity of trimethoprim *in vivo* has been poorly defined. *In vitro* experiments on *E. coli* showed that the presence of thymine or thymidine in the growth media antagonises the activity of trimethoprim. If minimal salts medium is used, a rapid halt in the synthesis of DNA, RNA and proteins is observed, even at high trimethoprim concentrations. The halt in macromolecule synthesis does not significantly affect the viability of the cells, suggesting that under these conditions, the action of trimethoprim is bacteriostatic (Then and Angehrn 1973). When the minimal salts medium is supplemented with the amino acids methionine and glycine plus a purine nucleoside, there is a rapid decrease in the viability of the cells, and trimethoprim becomes bactericidal (Amyes and Smith 1974a).

The cells undergo a phenomenon that has been described by Cohn (1971) as thymineless death. As a result of thymine starvation, the cell replaces thymine with uracil bases. The subsequent excision of these bases may lead to DNA fragmentation and cell death (Ayusawa *et al* 1983). The exact quantities of thymine, methionine, glycine and purines available in biological fluids is difficult to determine. Since methionine, glycine and purines are exogenously available, it can be assumed that cell death occurs as a result of thymine starvation (Then and Angehrn 1973).

1.3 Rationale behind co-trimoxazole

Daniel and co-workers (1947) found that analogues of pteridines such as certain diaminopyrimidines possess antibacterial activity which is antagonised by folic acid. Potter (1951) showed that two inhibitors which act on different enzymes of the same metabolic sequence can produce a greater inhibitory effect when used in combination than the sum of the inhibitory effect of the individual inhibitors. This phenomenon was explained as the sequential blockade of a metabolic pathway. Hitching's group realised the close similarity between the antimetabolite action of analogues of pterins and para-amino benzoic acid (Figure 1.5). They therefore pursued the sequential blockade concept with these two inhibitors where sulphonamides inhibited the biosynthesis of folic acid and diaminopyrimidines prevented the conversion of dihydrofolate to tetrahydrofolate. Synergy could be demonstrated when sub-inhibitory doses of diaminopyrimidines and sulphonamides were combined, they were capable of inhibiting cell growth. The combination was shown to be so potent *in vitro* that about one tenth as much of each moiety, in terms of effective dose was required for growth inhibition (Elion *et al* 1954).



p-amino benzoic acid sulphamethoxazole

Figure 1.5. Structure of p-amino benzoic acid and sulphamethoxazole.

Bushby and Hitchings (1968) intensively studied the synergy between different sulphonamides and DHFR inhibitors. For antibacterial use they selected trimethoprim as a potentiator of sulphonamides because of its broad spectrum of antibacterial activity, a high degree of selectivity for bacterial dihydrofolate reductase and suitable pharmacodynamic and pharmacokinetic properties. Several sulphonamides were studied *in vitro* and *in vivo* and eventually sulphamethoxazole was chosen as the sulpha-compound which matched the half life of trimethoprim.

Several points argued in favour of the combination. Species such as *Bordetella, Haemophilus* and *Neisseria* which show borderline sensitivity to the individual drugs are more susceptible to the combination. The greater potency of the combination may

be regarded as greater effectiveness at the higher dose levels, or as a reduction in the potential side-effects made possible by diminishing the doses. The combination is bactericidal where the individual components are bacteriostatic. The combination was said to be less vulnerable to the development of resistant strains than the individual components (Bushby and Hitchings 1968). Initially, trimethoprim was recommended for use only in a fixed 1:5 ratio with sulphamethoxazole (Brumfitt *et al* 1973). However since the development of co-trimoxazole, the combination concept has been challenged.

1.4 Against co-trimoxazole

Although trimethoprim was released as a potentiator of sulphamethoxazole (Bushby and Hitchings 1968), it was shown that trimethoprim was an effective agent on its own (Brumfitt *et al* 1973). Synergy occurs *in vitro* at an optimum ratio of one part trimethoprim to 20 parts sulphamethoxazole (Bushby and Hitchings 1968). *In vivo* synergy is unlikely to occur as results show that the 1:5 commercial ratio produces a 1:20 ratio only in the blood, and for a limited time only. In tissue and sputum, trimethoprim has much better penetration than sulphamethoxazole and reaches concentrations higher than the MIC of susceptible pathogens (Brumfitt and Hamilton-Miller 1993). The concentration of sulphamethoxazole in sputum was shown to be one tenth of that reached in plasma (Brumfitt *et al* 1973). In these situations, synergy as a result of the sulphonamide moiety is unlikely to contribute to the inhibitory effects of trimethoprim (Lacey 1979). In urine, the ratio produced by the combination of the two drugs is 1:1 where the trimethoprim concentration far exceeds the MIC for susceptible pathogens, to the extent that no clinically significant synergy can occur (Brumfitt and Hamilton-Miller 1993). In urine, the action of co-trimoxazole has been shown to be bacteriostatic (Lewis *et al* 1974). Greenwood (1979) showed in an experimental model of the bladder that sulphamethoxazole does not contribute to the activity of trimethoprim. Clinical results have shown that there are no significant advantages in using the combination over trimethoprim in infections of the urinary and respiratory tracts (Brumfitt and Pursell 1972; Kasanen and Sundquist 1982; Amyes *et al* 1986a). The exception where co-trimoxazole may be an advantage is in the treatment of organisms which are moderately susceptible or resistant to trimethoprim e.g. *Pneumocystis carinii, Streptococcus pneumoniae*, *Neisseria* spp., *Brucella* spp., and *Moraxella.* (Csonka 1969; Lacey 1982; Brumfitt and Hamilton-Miller 1993). For most of these species co-trimoxazole is not usually the therapy of choice. Attempts to improve co-trimoxazole by changing the pharmacokinetics of the sulphonamide component have not been a commercial success (Brumfitt and Hamilton-Miller 1993).

Poe (1976) suggested that synergy between the two agents was not the result of a sequential blockade of the folic acid pathway, as suggested by Burchall (1979), but rather the simultaneous binding of the two agents to the *E. coli* DHFR. Then (1977) showed that p-amino benzoic acid at high concentrations abolished the synergy between the two components; however, Lacey (1979) showed that synergy still does occur at high sulphonamide concentrations in the presence of p-amino benzoic acid. Lacey (1979) also found that synergy did not occur in isolates that were resistant to

sulphonamides. The underlying mechanism of resistance to sulphonamides in the isolates tested was not determined. These conflicting results suggest that sulphonamides may have two points of action in producing synergy.

The claim that the diminished doses of each constituent reduces adverse side effects has been challenged. Clinical experience has shown that the most common side effect (allergic skin reaction) occurs in 3-13% of patients taking co-trimoxazole, most of these occurred as a result of the sulphonamide moiety. While most of these side effects are mild, there is a risk of progression to erythema multiforme and toxic epidermal necrolysis with fatal results. This aspect has recently received significant publicity. Haematological and hepatotoxicity have also been recognised as side effects of sulphonamides. Most patients with these side effects can take trimethoprim on its own. There are also more drug interactions associated with sulphonamides than with trimethoprim (Brumfitt and Hamilton-Miller 1993).

The claim that co-trimoxazole will slow down the emergence of resistance does not seem to have taken place. In Finland, the first country where trimethoprim was approved as an agent on its own, resistance did not emerge more rapidly than other parts of Europe (Kasanen and Sundquist 1982; Huovinen *et al* 1982; Huovinen *et al* 1985b). There was no significant increase in the incidence of trimethoprim resistance in other countries after trimethoprim was released as a single agent (Brumfitt *et al* 1983). The high incidence of sulphonamide resistance (25% to >50% in most countries) suggests that sulphamethoxazole can do little to suppress the emergence of resistance to trimethoprim (Grüneberg 1979; Hamilton-Miller 1979; O'Brien *et al* 1982).

1.5 Mechanisms of resistance to trimethoprim

The development of resistance as the response of bacteria to a challenge by antimicrobial agents is inevitable, trimethoprim being no exception. Trimethoprim is a chemically synthesised molecule, and no naturally occurring substance has been found that mimics its action. Therefore it seems unlikely that a resistance mechanism to trimethoprim has evolved specifically in the past (Kotch 1981). A number of mechanisms of resistance to trimethoprim have been described in organisms intrinsically resistant to trimethoprim. Anaerobes such as *Bacteroides* and *Clostridium* produce a chromosomal DHFR which is insensitive to inhibition by trimethoprim (Then and Angehern 1979). Species such as *Pseudomonas aeruginosa* have been shown to be resistant to a number of antimicrobials including trimethoprim, as a result of the poor penetration of the agent through the cell envelope (Grey and Hamilton-Miller 1977). Degradation of trimethoprim by the ruminal flora of cows has been demonstrated by Nielsen *et al* (1978). Most of the attention has been focused on the acquired mechanisms of resistance by organisms which are intrinsically sensitive to trimethoprim.

1.5.1 Thymineless mutants

Thymidylate synthase is responsible for the conversion of deoxyuridine monophosphate to thymine and in the reaction tetrahydrofolate is oxidised to dihydrofolate. This reaction creates a drain on the pool of tetrahydrofolate cofactors in the cell. A deficiency in the activity of thymidylate synthase has a dramatic cofactor sparing effect which greatly reduces the demand for the activity of DHFR. Such mutants are highly resistant to DHFR inhibitors such as aminopterin and trimethoprim, but are dependent on an external supply of thymine and thymidine for growth (Amyes and Smith 1975). These mutants arise readily *in vitro* in the presence of thymine and trimethoprim (Stacey and Simson 1965). It was thought that these mutants would be unlikely to arise during trimethoprim therapy as a result of the low levels of thymine in blood and in body tissue, however these mutants have been reported during therapy on a number of occasions (King *et al* 1983; Maskell *et al* 1977; Platt *et al* 1983). Studies by Jobanputra and Datta (1974), Grey *et al* (1979) and Acar and Goldstein (1982) showed that these mutants accounted for less than 2% of trimethoprim-resistant isolates, suggesting that the overall clinical significance of these mutants is low. These mutants are usually associated with trimethoprim and or sulphonamide therapy and do not grow on Mueller-Hinton agar and growth on

MacConkey agar is variable (King *et al* 1983). Medium lacking in thymine or thymidine which is suitable for trimethoprim sensitivity testing is unable to support the growth of these mutants (Amyes and Smith 1974a; King *et al* 1983).

1.5.2 Impermeability

Resistance to trimethoprim can result from the acquisition of a mutation which decreases the permeability of the cell to trimethoprim. This mechanism of resistance appears to be common, however there is little evidence to support this, since isolates which fail to show changes in the nature or the expression of DHFR are often relegated to this category despite the failure to detect such a mechanism (Thomson 1993). Acar and Goldstein (1982) reported clinical isolates which showed cross resistance to trimethoprim, nalidixic acid and chloramphenicol in *Klebsiella*, *Enterobacter* and *Serratia* spp. Similar mutants displaying resistance to all three agents could be isolated *in vitro* by one-step selection procedures. These mutants showed reduced uptake of glucose and chloramphenicol; however no differences in DHFR expression were found, and binding of chloramphenicol to ribosomes was normal. Examination of the outer membrane proteins showed a reduction in the amount of a major uptake protein of about 40KDa (Gutmann *et al* 1985). The MIC range of trimethoprim in these isolates was between 128-256mg/l. Mutants showing cross resistance to trimethoprim and nalidixic acid have also been recorded by Then and Herman (1981). Williams Smith (1976) showed that these mutants arose in 27 of 32 epidemiologically unrelated strains of *Klebsiella pneumoniae* belonging to 20 different capsular types. These mutants arose in chicks that were experimentally infected with these organisms and then fed on antibiotic containing diets. These data suggest that these mutants may have clinical significance.

1.5.3 Alterations in chromosomal DHFR

Most of the earlier work done on the mechanisms of resistance to antifolates involved methotrexate resistance. Although there is some cross resistance between methotrexate and trimethoprim, this has been poorly studied (Hamilton-Miller 1979). *In vitro* mutants resistant to methotrexate showed an overproduction of DHFR in *E. coli* (Poe *et al* 1972) and in *Streptococcus pneumoniae* (McCuen and Sirotnak 1974). Similar mutants were selected using trimethoprim in *E. coli* K12 (Baccanari *et al* 1975). Sheldon and Brenner (1976) showed that chromosomally located, low-level resistance to trimethoprim could be induced *in vitro* in *E. coli*: resistance was due

either to a mutationally altered dihydrofolate reductase with a decreased sensitivity for trimethoprim or to a mutational change in regulation leading to increased levels of the chromosomal enzyme. In a survey conducted by Grey *et al* (1979) of 34 clinical strains (11 *E. coli*, 16 *K. aerogenes* and 7 *P. mirabilis*) which were not carrying R-factors conferring transferable trimethoprim resistance, most of the resistance was due to the production of DHFR with a reduced susceptibility to trimethoprim. In some isolates there was an increase in the specific activity of DHFR which was sufficient to account for the levels of resistance in these isolates. These isolates had intermediate levels of resistance, with trimethoprim MICs 5-500mg/l.

In a clinical survey (Tennhammer-Ekman and Sköld 1979), several bacterial strains were found that showed high-level resistance to trimethoprim, and this resistance was not plasmid borne. These strains had the common property of overproducing DHFR. One of these strains was further studied and showed an approximately 200-fold overproduction of DHFR activity. The restriction mapping and DNA hybridisation showed that the enzyme was similar to the *E. coli* K12 chromosomal DHFR but it differed in that it had a slower electrophoretic mobility, the K_i value for trimethoprim was three times higher and the turnover rate was 1.4 times higher. This isolate was also characterised by a 15-fold increase in the production of DHFR mRNA (Flensburg and Sköld 1984). The amount of enzyme present was shown to be inducible, and increased with increasing trimethoprim concentrations (6-fold increase at 100mg/l). The induction mechanism was dependent on protein synthesis and could not be induced by other folate analogues or thymine starvation (Tennhammer-Ekman *et al* 1986). DNA sequence analysis of this DHFR revealed two amino acid changes compared to the *E. coli* K12 chromosomal DHFR sequenced by Smith and Calvo (1980), a glycine residue was substituted for tryptophan at position 30 and a glutamine for glutamic acid substitution occurred at position 158. The amino acid change at position 30 which occurs within the active site of the DHFR is thought to relate to the increase in K_i of the DHFR. Furthermore, a cytosine to thymine transition was found in the -35 region of the promoter, increasing its homology with the *E. coli* consensus promoter sequence. In the ribosome binding area of the resistant strain, seven base changes were observed, two of which resulted in a five-base sequence of complementarity with the 3'-end of ribosomal 16S RNA. These changes are thought to increase the transcription and translation of the DHFR (Flensburg and Sköld 1987).

More recently, clinical strains of *Haemophilus influenzae* were isolated which expressed intermediate levels of resistance to trimethoprim (MIC 10-200mg/l) (de Groot *et al* 1988). Nucleotide sequence comparisons between resistant and susceptible isolates showed changes in the -35 promoter sequence making it homologous to that of *E. coli* mutants overproducing DHFR. Changes in the structural gene were also observed, glutamic acid was substituted for a lysine residue at position 69 and aspartic acid was substituted for an asparagine residue at position 77 (de Groot *et al* 1991).

It appears that mutants with altered chromosomal DHFR are restricted to clinical environments where there is strong selection pressure for trimethoprim resistance. Their occurrence in *Enterobacteriaceae* is not often clinically significant and is of lesser importance than plasmid-mediated resistance (Steen and Sköld 1985).

1.5.4 Production of an additional resistant DHFR

The production of an additional DHFR which is less sensitive to inhibition by trimethoprim than the chromosomal DHFR (Amyes and Smith 1974b; Sköld and Widh 1974) is by far the most important mechanism of resistance to trimethoprim. Plasmids carrying these genes were first identified by Fleming *et al* (1972) and were shown to confer high-level resistance to trimethoprim (MIC $\geq$1000mg/l). Since many of these resistant DHFR genes are located on transferable plasmids, this mechanism of resistance was originally referred to as plasmid-borne resistance, however this is not altogether true, since many of these genes are located on the chromosome as a result of transposase activity (Heikkilä *et al* 1991). To date, 16 of these DHFR genes have been identified and the biochemical properties, DNA sequence and/or amino acid sequence for each of these have been determined. The biochemical properties of these enzymes are shown in table 1.3.

Pattishall *et al* (1977) distinguished two different types of plasmid-encoded enzyme which could be distinguished entirely by their biochemical properties. The type I enzymes were produced in greater quantity and were 10 000 times more resistant than the chromosomal enzyme. The type II enzymes were produced in similar quantities to the chromosomal enzyme and are >1000 times more resistant than the type I enzymes. Following the subsequent discovery of the type III DHFR (Anderson 1980) which expressed intermediate levels of resistance (MIC 64mg/l) and the type IV DHFR which was inducible (Young and Amyes 1986a), all the DHFR types were still

Table 1.3. Biochemical properties of trimethoprim-resistant DHFR enzymes.

Enzyme	Tp ID_{50} μM	Mtx ID_{50} μM	TD_{50} min	DHF K_m μM	TP K_i μM	Size KDa #	Amino acids	MIC mg/l	
Family 1									
Ia	57.0	4.4	0.5	5.6	7.4	35	157	>1000	a
Ib	32.0	2.8	1.2	11.0	41	24.5	157	>1000	b
V	23.0	3.5	*	15.5	3.2	5	157	>1000	c
VI	200.0	7.3	0.4	31.2	75.0	10	157	>1000	d
VII	30.0	3.0	1.5	20.0	7.0	11.5	157	>1000	e
Family 2									
IIa	70 000	1100	>15	4.1	6100	37	78	>2000	f
IIb	80 000	750	>15	8.3	150	35	78	>1000	g
IIc	20 000	1000	>15	4.2	400	34	78	>1000	h
Ungrouped									
IIIa	2.0	0.02	>12	0.4	0.019	16.9	162	64	i
IIIb	2.0	0.02	>12	9.5	0.4	17	-	128	j
IIIc/VIII	3.0	0.007	8	3.1	0.5	22	169	256	k
IV	0.2	0.02	>12	37.0	0.063	46.7	144	10	l
IX	20	-	-	-	-	19.9	178	250	m
X	-	-	-	-	-	21.2	182	500	n
XII	700	-	-	-	-	-	165	>2000	o
S1	50.0	0.002	>12	10.8	11.6	19.7	161	>1000	p
E. coli K12	0.007	0.006	>12	3.2	0.0004	22	159	0.5	q

#As determined by sephadex gel filtration. *Dependent on protein concentration. a Pattishall *et al* (1977); b Young and Amyes (1985); c Thomson and Amyes (1988); d Wylie *et al* (1988); e Amyes *et al* (1989); f,g,h Pattishall *et al* (1977); Amyes and Smith (1978); Broad and Smith (1982); i Joyner *et al* (1984); j,k Barg *et al* (1990); l Young and Amyes (1986a); Tait (1993); m Jansson and Sköld (1991); n Parsons *et al* (1991); o Heikkilä *et al* (1993); p Young *et al* (1987); q Baccanari *et al* (1975); Pattishall *et al* (1977).

distinguishable by their biochemical properties. However the subsequent recognition of DHFR types Ib, IIIb, IIIc, V, VI, VII, VIII, IX, X and XII and the elucidation of the nucleotide and amino acid sequence of these DHFR genes created a dilemma with the original classification system based on biochemical properties alone (Amyes *et al* 1992b). For example, the type IIIb and IIIc DHFRs, although originally classed as subtypes of the type III, they share less than 50% amino acid identity with each other despite their similar biochemical properties. With the exception of the type IIIc DHFR which is identical to and has been renamed the type VIII (Barg *et al* 1995) the subtypes (Ib, IIa, IIb, IIc, IIIb) retain their original names (Amyes *et al* 1992b), furthermore, the type I has been renamed the type Ia so as to avoid confusion with the type Ib, and likewise, the type III is referred to as the type IIIa (Amyes and Towner 1990). The trimethoprim-resistant DHFRs have now been reclassified based on their nucleotide and amino acid sequence. Phylogenetic analysis has revealed that two subgroups of related trimethoprim-resistant DHFRs occur (Huovinen *et al* 1995).

1.5.4.1 Family 1 dihydrofolate reductases

The first group of enzymes, family 1 (type I like enzymes) includes enzyme types Ia (Fling and Richards 1983), Ib (Young *et al* 1994), V (Sundström *et al* 1988), VI (Wylie and Koornhof 1991) and VII (Sundström *et al* 1993). These enzymes share between 64% to 88% amino acid identity. Nucleotide sequence identity is the highest between the type Ib and V DHFR (88%) and ranges between 58-65% for the types Ia, VI and VII DHFRs (Young *et al* 1994). Table 1.6 shows the amino acid identity between the family 1 DHFRs.

Ia	100				
Ib	71	100			
V	75	88	100		
VI	63	64	61	100	
VII	71	66	66	64	100
	Ia	Ib	V	VI	VII

Figure 1.6. Percent amino acid identity between the family 1 DHFR enzymes.

These enzymes have a polypeptide length of 157 amino acids. The type Ib may be exceptional in that the nucleotide sequence reveals an open reading frame (ORF) of 160 amino acids between the AUG start codon at position 226, and the UAA stop codon at position 706. Sequence alignment with the types Ia, V, VI and VII DHFRs, suggests that the UUG codon at position 235 may function as an alternative initiation codon producing an ORF of 157 amino acids (Young *et al* 1994). A UUG triplet has been confirmed as the start codon of the type VII DHFR (Sundström *et al* 1993). The apparent molecular weights of these enzymes all appear to be different as determined by sephadex gel filtration and at least the type Ia is homodimeric (Novak *et al* 1983). All the enzymes in this class carry a glutamate corresponding to the aspartate residue at position 27 of the *E. coli* chromosomal enzyme (Sundström *et al* 1993). Since an aspartic acid residue is found within the folate binding site of the chromosomal DHFRs of trimethoprim susceptible bacteria, and a glutamate residue is found in the corresponding site of the DHFRs of vertebrates which are insusceptible to trimethoprim (Appleman *et al* 1990) it is thought that this may relate to the high trimethoprim MICs (>1000mg/l) mediated by this family of enzymes. These enzymes are all relatively unstable and lose ≥50 % of their activity in less than three minutes at 45°C. The stability of the type V and VI enzymes are dependent on background protein concentrations (Thomson and Amyes 1988; Wylie *et al* 1988). With the exception of the type VI which is more resistant (Wylie *et al* 1988), the ID_{50} for these enzymes for trimethoprim and methotrexate range from 20-57μM and 2.3-4.4μM respectively. The K_i values for these enzymes range from 7.0-75μM; the highest K_i was recorded for the type VI DHFR (Wylie *et al* 1988).

1.5.4.2 Family 2 dihydrofolate reductases

The second group, family 2 remains unchanged from the classification systems of Amyes and Towner (1990) and Amyes *et al* (1992b) which includes all the type II subtypes. The enzyme encoded by plasmid R67 is termed IIa (Stone and Smith 1979) and the type IIb and IIc dihydrofolate reductases are encoded by plasmids R388 (Swift *et al* 1981) and R751 (Flensburg and Steen 1986) respectively. These enzymes are remarkably different from other DHFRs in that they are virtually insensitive to antifolates including the broad spectrum DHFR inhibitor, methotrexate (Amyes and Smith 1976). These enzymes are unique in that they share no homology with other DHFRs, which are all similar in amino acid sequence, size, and active site residues. This suggests that these enzymes have completely different evolutionary origins (Swift *et al* 1981). The relative molecular weight of the type II enzymes as determined by gel

filtration suggest that they are tetrameric (Pattishall *et al* 1977; Amyes and Smith 1978). Matthews *et al* (1986) showed that the crystalline form of the DHFR produced by R67 is dimeric, however Nichols *et al* (1993) showed that for the pH range 5-8, tetrameric R67 DHFR reversibly dissociates into dimers, as monitored by ultracentrifugation and molecular sieving techniques. Modification of histidine residues stabilises dimeric R67 DHFR and causes a 200-600-fold decrease in catalytic efficiency. Decreased catalytic activity in dimeric R67 DHFR is presumably due to loss of the putative single active site pore found in tetrameric R67 DHFR (Nichols *et al* 1993). Stone and Smith (1979) suggested that an active site resembling that of a conventional DHFR may be formed as a result of the secondary structure formed by the active tetramers. However the complete lack of affinity of these enzymes to close folate analogues such as methotrexate suggest otherwise (Matthews *et al* 1986). An antibody which binds to all three type II enzymes does not cross react with the type Ia and T4 phage DHFR (Fling and Elwell 1980). It has been suggested that these enzymes may not be "classical" DHFRs, but rather a different type of oxidoreductase which has lost some degree of substrate specificity but has acquired the ability to reduce dihydrofolate as a consequence (Smith *et al* 1979; Matthews *et al* 1986). DNA and amino acid sequence of these enzymes have shown that they code for a polypeptide of 78 amino acids and share between 78 and 86% amino acid identity with each other (Stone and Smith 1979; Swift *et al* 1981; Brisson and Hohn 1984; Flensburg and Steen 1986). The C and N terminals of the monomers appear to be less conserved within this group (Brisson and Hohn 1984). The biochemical properties of these enzymes are summarised in table 1.3.

1.5.4.3 Ungrouped resistant dihydrofolate reductases

The remaining DHFR types IIIa, IIIb, IV, VIII, IX, X, XII and S1 share between 20 and 50% amino acid identity with each other and the *E. coli* chromosomal enzyme (Huovinen *et al* 1995). Similar levels of DHFR sequence homology occurs in the range 27-31% between different bacteria species, with higher levels of identity occurring between more closely related species (Fling *et al* 1988). There is therefore no phylogenetic basis by which these enzymes can be grouped. The biochemical properties of these enzymes are shown in table 1.3.

Type IIIa

The type IIIa was the first plasmid encoded DHFR conferring intermediate resistance to trimethoprim (MIC 64mg/l) to be discovered (Anderson 1980; Fling *et al* 1982). The enzyme is a monomer of 162 residues and is closely related to the *E. coli* chromosomal enzyme: the nucleotide sequence shares 57% identity, with some short stretches (11/12 bases) of perfect match (Fling *et al* 1988). Kinetic analysis showed that trimethoprim binds quite tightly to the enzyme (with a K_i= 0.019μM which is about 50 times less than the *E. coli* chromosomal enzyme), however the isolates are about 320-fold more resistant. Since the fractional activity of an enzyme in the presence of a competitive inhibitor is dependent upon both the I/K_i and S/K_m ratios, the level of resistance is thought to occur as a combination of the enzyme's 8-fold greater affinity for dihydrofolate (K_m=0.4μM) together with the 50-fold reduced sensitivity to trimethoprim. The specific activity of the enzyme produced by plasmid pAZ1 is 75% of that of the host's enzyme and is therefore unlikely to contribute significantly to resistance levels in the host (Joyner *et al* 1984).

Type IIIb

The type IIIb DHFR shares almost identical biochemical properties with the type IIIa, and was originally thought to be a subtype. This enzyme differs from the type IIIa in that it has a higher K_i and a different isoelectric point (Barg *et al* 1990). Subsequent N-terminal amino acid sequence analysis has revealed that the proposed relationship with the type IIIa is superficial. Amino acid identity with the type IIIa for the first 47 residues is 31%. Most of the identical amino acids occur within the conserved dihydrofolate binding sites of the enzymes (Thomson *et al* 1990a).

Type IIIc/VIII

The type IIIc is another enzyme which confers intermediate trimethoprim MICs, and shares very similar biochemical properties to the type IIIa and IIIb enzymes. This enzyme is extremely sensitive to inhibition by methotrexate, but is slightly more resistant to trimethoprim than the types IIIa and IIIb (Barg *et al* 1990). Subsequent nucleotide sequence analysis has revealed it to be identical to the type VIII DHFR (Sundström *et al* 1991; Barg *et al* 1995). Since it shares only 30% identity with the type IIIa, the subtype name has been dropped and it has been renamed the type VIII (Barg *et al* 1995).

Type IV

The type IV is an unusual DHFR in that the expression of its gene product is dependent on the concentrations of methionine, glycine and purines in the growth medium. In complex media containing these nutrients, the expression of the resistant DHFR is suppressed: MIC=5mg/l on Mueller-Hinton (Difco) and Iso-Sensitest agar (Oxoid). However the expression of this enzyme can be induced by a deficiency in these nutrients: in minimal medium the trimethoprim MIC is 160mg/l (Young and Amyes 1986a). Increasing trimethoprim concentrations were shown to reduce the number of viable cells, but increase the expression of the enzyme in the surviving cells. The enzyme is only twice as resistant as the *E. coli* chromosomal DHFR (trimethoprim ID_{50} =0.2μM) and has a low affinity for dihydrofolate (K_m=37): as a result, the suggested mechanism of resistance is said to be similar to that of mutants which over-express the chromosomal DHFR (Young *et al* 1986a). The enzyme produced by the clinical plasmid was thought to be a monomer and was shown to have a molecular weight of 46.7KDa by gel filtration and non-denaturing PAGE. However under SDS denaturing conditions, the purified protein revealed a major band of 33KDa and several minor bands which formed multiples of 10KDa. The N-terminal amino acid sequence of the purified protein revealed two polypeptides, the first 50 residues of the one polypeptide closely resembled those of the *E. coli* chromosomal DHFR (40% identity); the second polypeptide was identical to the NS1 DNA binding protein. This suggests that the protein produced by the clinical isolate is a heterodimer of these two proteins. A clone of the DHFR was shown to be active without the presence of the NS1 DNA binding protein (Thomson *et al* 1990b). Subsequent nucleotide sequence analysis of this clone has shown that the enzyme shares 26% amino acid homology with the chromosomal DHFR of *E. coli*. Amino acid homology with other resistant DHFRs is also low, and ranges between 19 and 30 percent (Tait 1993).

Type IX

The type IX DHFR also confers intermediate levels of resistance to trimethoprim (MIC 250mg/l) but its biochemical properties are unlike the type III like enzymes in that it has a significantly higher ID_{50} (20μM) for trimethoprim. This is almost in the ID_{50} range of the family 1 enzymes- the lower MIC conferred by the type IX could be explained by the low intracellular levels of the enzyme produced. The amino acid sequence of 178 residues shares only 26% similarity with the type I and the *E. coli* chromosomal DHFR (Jansson and Sköld 1991).

Type X

The type X DHFR confers an intermediate MIC for trimethoprim of 500mg/l. The biochemical properties of this enzyme have not been determined, however nucleotide sequence analysis has revealed that it is unrelated to other DHFR types (Parsons *et al* 1991).

Type XII

The type XII DHFR is unique in that it confers similar levels of resistance (MIC≥2000mg/l; ID_{50} =700μM) to the atypical type II enzymes, but still shares close homology with other DHFRs in terms of structure and substrate binding sites (Heikkilä *et al* 1993). With the exception of the family 1 enzymes, the type XII DHFR is the only other resistant DHFR to carry a glutamate corresponding to the aspartate residue at position 27 of the *E. coli* chromosomal enzyme (Huovinen *et al* 1995). The type XII DHFR shares between 32% and 39% amino acid identity with DHFR types I, III, V, VI and VII (Singh *et al* 1992b).

S1 DHFR

All the DHFR types have so far been detected in Gram-negative bacteria. It was not until 1983 that the first transferable trimethoprim resistance was detected in *Staphylococcus aureus* in Australia (Townsend *et al* 1984). A resistant DHFR was later identified as the mechanism of resistance. The enzyme confers high-level trimethoprim resistance (MIC>1000mg/l) and is 1000-fold more resistant to trimethoprim (ID_{50}=50μM) than the chromosomal DHFR (ID_{50}=0.04μM). Unlike other resistant DHFRs, it does not confer resistance to methotrexate (Young *et al* 1987). Since this gene has been detected only in Staphylococci, it is designated the S1 DHFR. The nucleotide sequence of the S1 DHFR does not show close homology to other resistant DHFR types (Rouch *et al* 1989). The N-terminal amino acid sequence of the first 35 residues of the *S. aureus* chromosomal DHFR shares 80% identity with the S1 DHFR suggesting it may have evolved from the chromosomal DHFR (Burdeska *et al* 1990).

1.6 Molecular epidemiology of resistant DHFRs

DNA hybridisation provides a useful epidemiological tool to monitor the distribution and spread of antibiotic resistance genes in a population and to obtain information regarding the evolutionary relationships of these genes (Towner 1992). In most cases gene probes are constructed from cloned sections of the resistance gene. These probes are often relatively large in size (up to several kilobases) and are not often efficient at distinguishing between closely related genes (Towner 1992; Qumsieh and Young 1991). Since resistance genes often share similar flanking sequences such as those of integrons and IS-elements, gene probes containing these overlapping regions have the potential to cross-hybridise, and as a result overestimate the frequency of a resistance gene in a population (Sundström *et al* 1987; Agodi *et al* 1990). These problems can be overcome by synthesising small oligonucleotide probes that are capable of recognising single base differences (Mabilat and Courvalin 1990).

A number of hybridisation studies have been done to determine the distribution of resistant DHFR genes at various centres. Comparisons between these data are difficult because of discrepancies in sample collection and differences in the probes and hybridisation methods used. The gene probes for many of the more recently discovered DHFR types are often not available and as a result few of these studies are comprehensive. Table 1.4 shows the incidence of trimethoprim-resistant DHFRs from studies at various centres around the world.

Type Ia

The type Ia DHFR is by far the most widely distributed trimethoprim-resistant DHFR, and has been detected in most collections of resistant isolates (Table 1.4). This enzyme has been shown to occur in more than 50% of resistant isolates in Sweden, Finland, France and the UK (Figure 1.7). However at centres outside Europe, the type Ia DHFR appears to account for a smaller percentage of resistant isolates.

Table 1.4. Incidence of resistant DHFRs at various centres around the world.

			DHFR types %								
Country	Isolates	year	Ia	II	IIIa	IV	V	VII	Other	N. H.	
Nottingham UK	Urinary plasmids	1978-1983	86	0	1	0	10	1	-	1	a
Nottingham UK	Urinary plasmids	1987-1988	62	1	20	0	6	7.2	-	3	b
Nottingham UK	Urinary plasmids	1992	59	0	3	0	25	0	-	13	c
Paris, France	Enterobacteria plasmids	1972-1984	49	36*	-	-	-	-	-	30	d
Paris, France	Clinical Enterobacteria	1981-1984	60	35*	-	-	-	-	-	15	e
Finland	Urinary out-patients	1984-1988	57	3	0	-	0.5	-	-	41	f
Turku, Finland	Urinary	1984-1988	70	0.5	0	-	0	1	-	29	g
Finish Travellers	*Shigella* spp.	1985-1988	85	0	0	-	3	-	-	12	h
Sweden	Porcine *E. coli*	1984-89	59	2	-	-	0	0	IX 11	10	i
Greece	Urinary *Enterobacter*	1991	18	58	-	-	2	-	-	32	j
Greece	Urinary *E. coli*	1991	44	19	-	-	2	-	-	41	k
Sicily, Italy	*S. sonnei*	1985-87	0	0	-	0	38	-	-	63	l
USA	*S. sonnei*	1985-1987	27	0	0	-	-	-	IIIc 73 IIIb 13	0	m
Texas, USA	Faecal *E. coli*	1991	17	0	0	-	4	0	XII 21	68	n
various	Clinical Enterobacteria	1972-1982	52	38	-	-	-	-	-	21	o
Taiwan	Urinary	1987-89	45	0	0	0	10	-	-	45	p
Perth, Australia	*E. coli*	1985	0	100*	-	-	-	-		0	q
South Africa	Urinary Enterobacteria	1986-1987	22	0	-	-	-	-	-	78	r
Sri Lanka	Enterobacteria plasmids	1985	5	0	0	-	≥9	-	-	-	s
Vellore, India	Faecal plasmids	1990	31	0	0	19	36	0	Ib 14	0	t

(N.H.) Percentage of isolates which did not hybridise to any of the DHFR probes; (-) Isolates not tested with this probe; (*) Type II DHFR probe >700bp; a Towner *et al* (1991); b Towner *et al* (1991); c Towner *et al* (1994); d Goldstein *et al* (1986); e Papadopoulou *et al* (1986); f Heikkilä *et al* (1990a); g Heikkilä *et al* (1990b); h Heikkilä *et al* (1990c); i Jansson *et al* (1992); j Tsakris *et al* (1992); k Tsakris *et al* (1993); l Agodi *et al* (1990); m Barg *et al* (1990); n Singh *et al* (1992b); o Fling *et al* (1982); p Chang *et al* (1992); q Campbell *et al* (1986); r Wylie and Koornhof (1989); s Sundström *et al* (1987); t Tait and Amyes (1994).

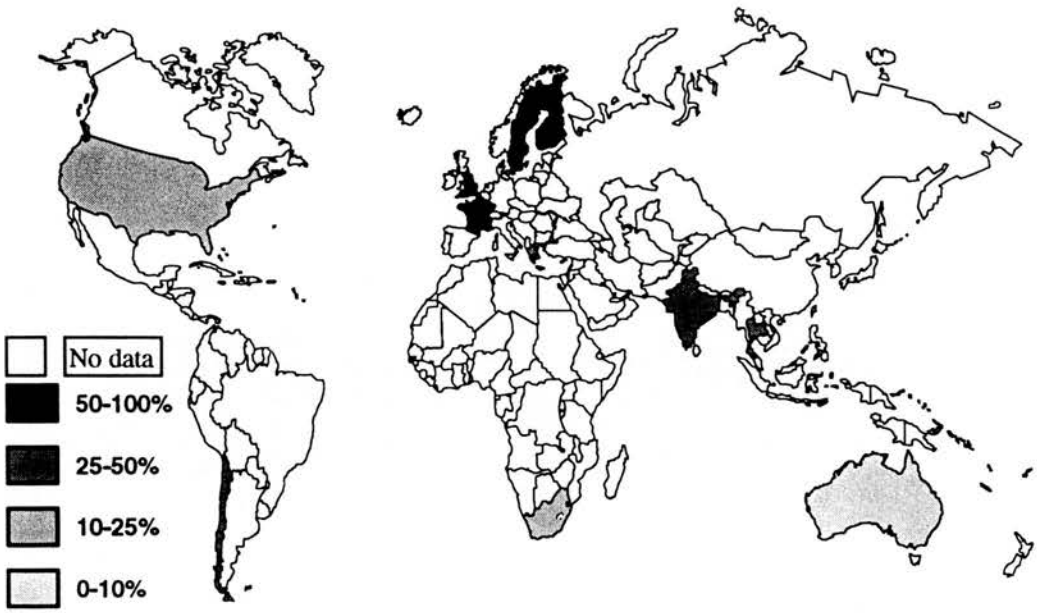


Figure 1.7. Prevalence of the type Ia DHFR in various countries.

The type Ia DHFR was first detected in a urinary *E. coli* isolate from a London Hospital and was located on plasmid R483 (Jobanputra and Datta 1974). Barth *et al* (1976) demonstrated that a segment of DNA from R483 could be transposed onto plasmids or onto the chromosome and postulated that the mobile element was a transposon (TnC, renamed Tn*7*) which had distinct genetic boundaries. Identical elements were later demonstrated to occur within different clinical plasmids (Barth and Datta 1977). The majority of isolates harbouring the type Ia DHFR, have been shown to hybridise positively to a Tn*7* specific probe (Steen and Sköld 1985; Heikkilä *et al* 1991; Jansson *et al* 1992; Tsakris *et al* 1993; Towner *et al* 1994). Much of the success of the type Ia DHFR is based on its ability to integrate as part of Tn*7* into the attTn*7* site on the chromosome of *E. coli* and other bacteria (Lichtenstein and Brenner 1981). This mechanism is believed to account for the increase in non-transferable high-level resistance observed by Towner *et al* (1982), Amyes *et al* (1986b) and Papadopoulou *et al* (1986).

The type Ia DHFR is found as part of a cassette-like structure thus giving it the potential to be inserted by integrase activity into integron-like structures of different genetic elements. The type Ia DHFR has been found within integron structures of Tn*7* (Sundström *et al* 1991b) and the antimicrobial resistance integron of the predominantly plasmid located transposon Tn*21* (Sundström and Sköld 1990). A study by Heikkilä *et al* (1991) found the type Ia DHFR on the chromosome in 75 out

of 76 isolates. Nine of these isolates carried the type Ia DHFR on both the plasmid and chromosome and only one isolate carried the gene on a plasmid only. Of these isolates, 61 (80%) probed positive for the *tns*C gene suggesting an active Tn*7*. Fifty of these isolates (82%) had Tn*7* located in the attTn*7* site.

Type Ib

The type Ib DHFR was first detected harboured on clinical plasmid pUK163 which was isolated from a urinary pathogen isolated in Edinburgh. The DHFR gene occurred on Tn*4132*, a Tn*7* related transposon which could be mobilised onto plasmid RP4 (Young and Amyes 1985). The nucleotide sequence of the 3' end of the gene was identical to the 59-base element which is present downstream of the type V DHFR (Young *et al* 1994) and is believed to play a role in the site-specific insertion of gene cassettes into integron structures. The 500-bp *Hinc*II fragment of pUK1224 developed as a specific gene probe for the type Ib enzyme by (Qumsieh and Young 1991), was shown to hybridise to the type V gene. An increase in the stringency of the washes reduced the level of cross hybridisation with this probe. Based on the sequence alignment of the types Ib and V DHFRs, oligonucleotide probes developed from the most dissimilar regions of these genes could clearly distinguish between the two (Young *et al* 1994). Subsequent hybridisation experiments using these probes showed that the type Ib enzyme was the predominant DHFR type in clinical plasmids from Dundee (Young *et al* 1994). Of the trimethoprim-resistant DHFRs from South India which were originally identified as being type V, 39% were subsequently shown to be type Ib enzymes (Tait and Amyes 1994). These data suggest that the type Ib is far more widespread than originally thought. The oligonucleotide probe for the type V DHFR described by Sundström *et al* (1993) differs from the type Ib by only one nucleotide, and is therefore unlikely to distinguish between these two genes.

Type V

The type V DHFR was first detected in a study of Sri Lankan Enterobacteria isolates (Sundström *et al* 1987). Forty-one out of 58 isolates were demonstrated to hybridise to a 2.6-kb *Ava*I fragment which harboured the type II DHFR from R388, however some of these isolates expressed uncharacteristically high intracellular levels of DHFR for a type II enzyme, and were more sensitive to trimethoprim. All the isolates failed to hybridise with a smaller 275-bp *Eco*RI-*Sau*3A1 fragment harbouring 153-bp of the 3' terminal of the type II DHFR. Restriction endonuclease analysis showed that the enzyme was different from previously characterised enzymes. Nucleotide sequencing of this DHFR revealed that the surrounding sequences of this enzyme were identical

to those of Tn*21* and R388 (Sundström *et al* 1988) which explains the high degree of cross-hybridisation with the larger gene probe. Hybridisation experiments with a 500-bp *Hinc*II fragment of pLK09 which contains the type V DHFR gene (Towner *et al* 1988) have shown that this enzyme is extremely ubiquitous and has been detected in most of the collections of isolates (Table 1.4). A proportion of the enzymes identified as type V with this probe may be mis-identified type Ib enzymes (Tait and Amyes 1994).

Type VI

The type VI DHFR was described in a urinary isolate of *Proteus mirabilis* from South Africa (Wylie *et al* 1988). The prevalence of this gene in South Africa and other parts of the world has not yet been determined. An intragenic oligonucleotide derived from the type VI DHFR gene sequence (Wylie and Koornhof 1991) failed to hybridise to isolates from Sweden, Finland, Nigeria and Sri Lanka (Sundström *et al* 1993).

Type VII

The type VII DHFR was first detected on plasmid pUN835 harboured in a veterinary *E. coli* isolate from Nottinghamshire, UK, and was distinguished from other DHFRs on the basis of its unique biochemical properties and its failure to hybridise to probes of other known resistant DHFRs (Amyes *et al* 1989). Sundström *et al* (1993) identified an identical gene integrated in a cassette-like manner on a Tn*21*-like transposon which has been designated Tn*5086*. A restriction map of the DHFR in pUN835 (Towner and Carter 1990) showed that it was identical to that of Tn*5086*. This gene was detected in Swedish clinical isolates from as early as 1974, and has subsequently been detected with an intragenic oligonucleotide probe in isolates from Finland, Nigeria and Sri Lanka (Sundström *et al* 1993).

Type II

Despite the widespread availability of the type II DHFR gene probes, the type II DHFRs have been absent or detected at low frequencies in most of the collections of isolates studied (Table 1.4). Since most of the resistant DHFR genes including those from R388 and R67 occur flanked by sequences identical to Tn*21* (Huovinen *et al* 1995), it has been demonstrated that DHFR gene probes that contain these flanking sequences can cross-hybridise with DHFR genes located within these surroundings (Sundström *et al* 1987; Agodi *et al* 1990). The data obtained by Fling *et al* (1982) and Campbell *et al* (1986) who detected a high incidence of the type II DHFR genes is unlikely to be reliable since a 2.5-kb *Eco*RI-*Bam*HI fragment of R67 was used for

the type II DHFR gene probe. A shorter 800-bp *Eco*RI fragment also detected a high incidence of this gene in French isolates (Papadopoulou *et al* 1986; Goldstein *et al* 1986). The 275-bp *Eco*RI-*Sau*3A1 fragment of pWZ820 (derived from R388) described by Towner *et al* (1991) and the 0.28-kb *Nhe*I-*Eco*RI fragment from plasmid R751 described Heikkilä *et al* (1990a) do not appear to cross-hybridise with other DHFRs.

Type IIIa

The type IIIa was first detected on a 5.2 MDa transferable plasmid from a strain of *Salmonella typhimurium* phage type 179 isolated in New Zealand (Anderson 1980) and in a *S. typhimurium* isolate of the same phage type in the United Kingdom (Fling *et al* 1982). This DHFR has subsequently been detected on a plasmid from a urinary *E. coli* isolate from Nottinghamshire (Thomson *et al* 1990c). A 700-bp *Eco*RI-*Pst*I fragment harbouring this DHFR was used as a gene probe to detect this gene in 20% and 1% of trimethoprim-resistant urinary *Enterobacteriaceae* isolated from 1987-1988 and 1992 in Nottingham respectively (Towner *et al* 1990; Towner *et al* 1994). Despite the widespread use of type IIIa DHFR gene probes, this DHFR has not been detected in most of the surveys conducted (Table 1.4).

Type IIIb

The type IIIb was first isolated from an outbreak of trimethoprim-resistant *S. sonnei* at a nursing home in the USA. A 1.6kb *Pst*I fragment harbouring this gene was shown to hybridise to one other isolate of *S. sonnei* from Hawaii (Barg *et al* 1990).

Type IV

The type IV DHFR gene has been detected on plasmids from both urine and faecal *E. coli* isolates from South India (Young *et al* 1986a; Tait and Amyes 1994). Gene probing with a 1.7kb *Cla*I fragment of pUK1148 as described by Towner *et al* (1988) showed that 19% of plasmids isolated from commensal faecal flora from this region harboured this gene (Tait and Amyes 1994).

Type IIIc/VIII

The type VIII DHFR appears to be widespread in the USA. CDC isolates of *Shigella sonnei* from eight different states hybridised to a probe constructed from a 1.8kb *Pst*I fragment harbouring the type VIII DHFR (Barg *et al* 1990). The type VIII has also been detected in Sweden in urinary isolates of *E. coli* (Sundström *et al* 1991a).

Type XI

This enzyme was first isolated from *E. coli* during an outbreak of porcine diarrhoea in Sweden (Jansson and Sköld 1991). Hybridisation experiments with an intragenic oligonucleotide probe showed that this gene occurred in 11% of trimethoprim-resistant veterinary isolates from Sweden. To monitor the prevalence of this gene in human isolates, resistant organisms from Sweden (222), Finland (46), Texas (14), Ethiopia (26), Nigeria (80) and Thailand (46) were probed: only one isolate from Sweden harboured this DHFR (Jansson *et al* 1993).

Type X

The type X DHFR was isolated during an outbreak of gentamicin resistant *E. coli* at a Sydney hospital. The DHFR gene was shown to be inserted in a cassette-like manner in the In7 integron on a large conjugative plasmid pDGO100 (Parsons *et al* 1991). This DHFR has not been detected in any other studies.

Type XII

The type XII DHFR was first detected in the USA in faecal isolates of *E. coli.* Subsequent hybridisation experiments with a 100-bp *Alu*I intragenic fragment detected the type XII gene in 21% of commensal faecal isolates from children attending day-care centres (Singh *et al* 1992b). Sequence analysis of a Finnish isolate harbouring the type XII DHFR revealed flanking sequences that differed from those surrounding the DHFR isolated in the USA (Heikkilä *et al* 1993) . The Finnish isolate was demonstrated to be cassette mediated, and was located within the integron of a Tn*21*-like element. Heikkilä *et al* (1993) showed that 2.7% (4/150) Finnish urinary *E. coli* isolates and 2.3% (4/175) isolates of *Shigella* spp. hybridised to an intragenic oligonucleotide probe for the type XII. Three of the *Shigella* isolates were isolated from travellers returning from Thailand and the fourth came from a Vietnamese immigrant. These two studies suggest that although this DHFR type is not highly prevalent, it is widely distributed amongst *Enterobacteriaceae.*

1.7 The commensal faecal flora: a reservoir of resistance

The gastro-intestinal tract is the largest reservoir of bacteria associated with the human body. The intestinal flora form a diverse and dynamic ecosystem consisting of numerous species of Gram-negative anaerobes, Gram-positive cocci, members of the *Enterobacteriaceae*, fungi and viruses (Tancrède 1992). The relationship between commensal bacteria and man appears to have many far reaching consequences. One of the positive aspects of gastrointestinal colonisation is the phenomenon of colonisation resistance (Tancrède 1992). Van der Waaij *et al* (1971) showed that the commensal flora play a role in preventing the colonisation of the gut by potentially pathogenic bacteria by competitive exclusion. The commensal faecal flora are also associated with disease. When the hosts defences are disrupted, for instance during neutropenia, members of the family of the *Enterobacteriaceae* can translocate and cause Gram-negative bacteremia (Tancrède and Andremont 1985). Anaerobes such as *Bacteroides* spp. are often isolated from blood cultures if the host's barriers are broken such as during bowel surgery. The faecal flora have also been implicated as the source of infection of the urinary tract (Brumfitt *et al* 1971; Bettelheim *et al* 1971).

There are extensive data on the prevalence of antimicrobial resistance in pathogens in hospitalised patients (O'Brien *et al* 1987); however little attention has been focused on the origins and reservoirs of these resistance factors in the open population (Hawkey 1986). The administration of antimicrobial agents is the most common cause of disturbances in the normal micro flora. It leads to the inhibition of sensitive commensal organisms followed by the selection and subsequent overgrowth of resistant strains. There is also potential for re-colonisation by exogenous resistant organisms (Datta *et al* 1971; Shaw *et al* 1973). There is also growing evidence to suggest that the commensal flora are the largest reservoir of antibiotic resistant determinants. Most of the surveys performed on resistance levels in commensal faecal flora have focused on resistance in the *Enterobacteriaceae*, *E. coli* being the most frequently isolated pathogen in hospital acquired infections (Bettelheim *et al* 1971; Huovinen *et al* 1982). Bacterial diarrhoeal disease caused predominantly by *Enterobacteriaceae* is also the greatest cause of mortality and morbidity in third world countries (Kunin *et al* 1987).

1.7.1. Epidemiology of resistance in commensal flora

Developed countries

Early studies showed that resistance carriage rates in Gram-negative aerobic commensal faecal flora were high. A study by Moorehouse (1969) of 100 Irish infants showed that 81% carried resistant strains. Datta (1969) found that 52% of pre-admission patients at a London hospital carried resistant coliforms. Resistant strains predominated in 28% of the patients. A similar study from a male urological ward in Denmark showed a 67% incidence of resistant *E. coli* in the bowel flora (Søgaard 1975). A study of adults and children from rural and urban populations in the United Kingdom showed that 55% carried resistant strains. Higher carriage rates were associated with children, and rural populations involved with animals (Linton *et al* 1972).

More recent studies from developed countries have shown that not only is the incidence of antimicrobial resistance in the commensal faecal flora increasing, but also the range of antimicrobials to which these organisms are resistant. There has also been an increase in the numbers of multi-resistant strains that have been isolated in commensal faecal flora. Studies from the Netherlands have shown that the incidence of resistance in commensal faecal flora was 66%. No differences in resistance levels between different age groups were detected (Degener *et al* 1983). Bonten *et al* (1990) showed that the incidence of resistance in faecal samples from Dutch students to sulphonamides, ampicillin, tetracycline and trimethoprim was 86%, 76%, 47% and 25% respectively. A further study by Bonten *et al* (1992) showed that the frequency of resistance in populations from two Dutch cites was as follows: in Maastricht, resistance to ampicillin, tetracycline, sulphonamides and trimethoprim occurred in 62%, 68%, 71%, and 45% of faecal isolates respectively, and in Zwolle resistance to ampicillin, tetracycline, sulphonamides and trimethoprim occurred in 89%, 49%, 49%, 42% of faecal isolates respectively. Seventy-seven percent of these isolates were resistant to more than one antimicrobial agent, 31% of which were resistant to five or more antimicrobial agents. A 15 week follow up survey conducted by London *et al* (1993) showed that there were no significant changes in the carriage rate of resistant flora in individuals over the study period. In a study of commensal faecal flora from healthy populations in Scotland, carriage rates of resistant organisms were 42%, 27%, 12% and 2% for ampicillin, tetracycline, trimethoprim and nalidixic acid respectively.

In the USA, Levy *et al* (1988) showed that 60% of healthy volunteers carried resistant flora. Resistant organisms dominated (>50%) the faecal flora in 34% of the volunteers. A more recent study by Lester *et al* (1990) showed that the faecal carriage rates of resistant organisms was about 54% in Boston. Resistance to trimethoprim was detected in 3% of the stools. Reves *et al* (1987) showed that in infants attending day-care centres in the United States the faecal carriage rates of ampicillin and trimethoprim resistance were 65% and 28% respectively. A further study by Reves *et al* (1990) showed that the faecal carriage rate of trimethoprim resistance was 30% in children attending day-care centres. This was significantly higher than in the control group of children (6%) and medical students (8%). These data suggest that the high levels of trimethoprim resistance in day care centres are due to the greater likelihood of the introduction of resistant organisms to the pool, the greater potential for spread due to the low standards of personal hygiene of the infants, and/or to prolonged survival of strains. Fornasini *et al* (1992) showed that family members of children attending day-care centres were at a greater risk of faecal colonisation by trimethoprim-resistant organisms. Table 1.5 shows the data for the incidence of resistance to individual antibiotics.

Developing countries

The situation in developing countries appears to be far more serious. Studies from China and Venezuela involving 41 and 53 healthy children respectively showed that faecal carriage of resistant organisms was almost universal. Resistance to trimethoprim occurred in 64% and 61% of healthy children respectively (Lester *et al* 1990). In India faecal carriage of organisms resistant to ampicillin, trimethoprim and chloramphenicol is almost universal (Amyes *et al* 1992a). In a one year study of commensal organisms in children from Bangladesh (Mamun *et al* 1993) the average incidence of antimicrobial resistance to the following drugs: ampicillin, trimethoprim, tetracycline, and sulphonamide was 72%, 45%, 65% and 62% respectively. In all but two months over 50% of the children were colonised with coliforms resistant to three or more antibiotics. The incidence of resistance was higher during the monsoon season, and it has been suggested that this is most likely due to the contamination of the water supply by resistant organisms during the wet season.

Table 1.5. Antimicrobial resistance in commensal faecal flora at different centres.

Survey	Percentage of isolates resistant to									Reference
	Ap	Tp	NA	Cm	Tc	Sx	Sm	Km	Gm	
Developed										
Ireland	62	-	0	16	76	-	-	63	-	Moorhouse (1969)
UK	17	-	-	8	34	38	27	1	-	Datta (1969)
UK	25	-	-	5	22	17	21	-	-	Linton *et al* (1972)
UK	48	-	-	-	61	-	-	-	-	Shaw *et al* (1973)
Denmark	18	-		9	46	54	53	-	-	Søgaard (1975)
Netherlands	26	-	-	-	42	46	-	-	-	Degener *et al* (1983)
Netherlands	76	25	-	-	47	86	-	-	-	Bonten *et al* (1990)
Netherlands	76	44	-	-	59	61		-	-	Bonten *et al* (1992)
USA	35	8	1	4	31	-	34	21	5	Levy *et al* (1988)
USA	23	3	-	15	33	36	38	8	-	Lester *et al* (1990)
Scotland	42	12	2		27				1	Shanahan *et al* (1994b)
Developing										
Venezuela	85	61	-	56	93	93	95	56	-	Lester *et al* (1990)
China	47	64	-	42	92	87	79	26	32	Lester *et al* (1990)
India	98	98	23	97	-	-	-	-	-	Amyes *et al* (1992)
South Africa	87	74	10	53	-	-	-	-	8	Shanahan *et al* (1993)
Bangladesh	72	45	-	63	65	62	78	-	-	Mamun *et al* (1993)

In South Africa, the levels of resistance in commensal faecal flora amongst the urbanised and rural black communities (ampicillin 87%, trimethoprim 74%, chloramphenicol 53%) appear to be similar to those of other developing countries (Shanahan *et al* 1993). The study populations were associated with a low standard of living and significant overcrowding; however South Africa is unique in that there is little unprescribed use of antimicrobial agents and the water supply is often uncontaminated, suggesting that these two factors need to be re-evaluated with respect to the role played in the spread of resistance factors in a community. It is worth noting that appropriately prescribed antimicrobials does not guarantee patient compliance (Shanahan *et al* 1994a).

1.7.2 Selection of resistant bacteria in faecal flora

Different antimicrobial agents have been shown to exert different selective pressure for drug resistance in the commensal faecal flora. Datta *et al* (1971) showed that in comparison to ampicillin and sulphonamides, tetracycline therapy had a greater effect on the selection and excretion of resistant organisms. It was shown that an increase in resistance to other antimicrobials after tetracycline therapy occurred as the result of the selection for multiply resistant strains. Lincoln *et al* (1970) showed that treatment with sulphonamides resulted in the disappearance of sulphonamide sensitive serotypes from the bowel followed by the emergence of resistant serotypes, some of which carried R-factors for multiple antibiotic resistance.

Trimethoprim is rapidly absorbed after oral administration and absorption from the gut is almost complete, as a result faecal concentrations of trimethoprim are low (Brumfitt *et al* 1969; Schwartz & Rieder 1970). A number of studies have investigated the effects of co-trimoxazole and trimethoprim therapy on the commensal faecal flora. A study by Brumfitt and Pursell (1972) and Brumfitt *et al* (1973) showed that trimethoprim and co-trimoxazole do not lead to the colonisation of the gut by resistant strains. These findings were supported by Pancoast *et al* (1980) who demonstrated that both therapies effectively cleared the introitus and rectal areas of *Enterobacteriaceae*. In the few individuals who had strains resistant to trimethoprim or co-trimoxazole before initiation of therapy, these organisms did not persist once therapy began. Guerrant *et al* (1981) showed that both trimethoprim and co-trimoxazole therapy resulted in a 4-log suppression of total coliforms during therapy. Both these regimens had no effect on the anaerobic commensal flora. During trimethoprim and co-trimoxazole therapy, resistant Gram-negative aerobes occurred in 58% and 22% of the patients respectively. Of the 158 resistant organisms isolated, only one incidence of a resistant *Enterobacteriaceae* was found. The majority of the resistant isolates were *Pseudomonas* and *Acinetobacter* species, which did not increase significantly in numbers during therapy.

A study by Stamey *et al* (1977) of the effects of long term trimethoprim therapy for the prophylaxis of urinary tract infections showed that therapy resulted in the elimination of *Enterobacteriaceae* from the gut and vaginal flora from most of the patients. *E. coli* were isolated from 27% of the faecal samples 8.8% of which were resistant to trimethoprim. Pearson *et al* (1979) found that during long-term treatment of urinary tract infection with co-trimoxazole, only 5% of resistant isolates examined

carried transferable trimethoprim resistance (MICs>1024mg/l). Reves *et al* (1990) showed that colonisation of trimethoprim-resistant flora in the USA is rare, even among individuals consuming trimethoprim. Contrary to these data, Huovinen *et al* (1985a) demonstrated that the incidence of resistant Enterobacteria in stool specimens increased three and four-fold during trimethoprim and co-trimoxazole therapy respectively: the original resistance levels were restored within one month after therapy.

Only one study has examined the effect of trimethoprim or co-trimoxazole therapy on the faecal flora in developing countries. In a study of American students taking prophylactic trimethoprim or co-trimoxazole on a visit to Mexico, Murray *et al* (1982) showed that trimethoprim and co-trimoxazole therapy did not change the numbers of total faecal *Enterobacteriaceae*. Virtually all the strains isolated expressed high-level trimethoprim and sulphamethoxazole resistance. In these isolates *E. coli* was the predominant organism, of which 95% carried resistance to at least four antimicrobial agents, and 25% were resistant to seven. Forty-one percent of these isolates carried transferable resistance; 60% of these shared the same epidemic plasmid (Rudy and Murray 1984). It appears that in areas where there is a high faecal carriage rate of resistant organisms especially those carrying transferable R-factors, therapy results in the disappearance of sensitive serotypes from the bowel followed by the emergence of resistant serotypes.

Antimicrobial agents which reach the lower bowel at significant concentrations in an active form are far more likely to select out resistant bacteria. A large proportion of ceftriaxone is biliarly excreted after injection. In a study with volunteers treated with 1g/d for 5 days with this antimicrobial, Léonard *et al* (1989) found that two different phenomena occurred. In one group, 1.8-2.0mg/g of ceftriaxone was detected in faeces and concomitantly, faecal counts of anaerobes dropped while those of *Candida* species increased 100-fold. In the second group, β-lactamase activity was high during ceftriaxone administration and as a result no ceftriaxone was detected in faeces. There were no significant changes in faecal counts of anaerobes or *Candida* species in this group.

Amyes and Gould (1984) showed that a change in prescribing policy at a small general hospital in Edinburgh from ampicillin to trimethoprim for the first line treatment of urinary tract infection resulted in a decrease in both ampicillin and trimethoprim resistance. It appears that ampicillin was therefore more effective at

selecting bacteria that were not only ampicillin resistant but trimethoprim-resistant as well. Since ampicillin is poorly absorbed in the gut, a considerable quantity of active drug reaches the large intestine which leads to strong selection pressure for resistant strains and results in the selection of multi-resistant strains. Reves *et al* (1990) suggested that trimethoprim resistance is an excellent indicator of the presence of multi-resistance plasmids and is a better indicator than ampicillin or sulphonamide resistance.

1.8 Clinical environment: a reservoir of resistance

The clinical environment is now a well-established reservoir of antibiotic-resistance genes. The extensive use of antimicrobials in clinical practice has created a high degree of selection pressure whereby bacterial strains resistant to a given antibiotic are selected for among strains being treated with that particular agent (Shanahan *et al* 1994a). When bacterial resistance data from different studies are compared, attention should be paid to sources of the isolates and the susceptibility testing methods used (Huovinen 1987). Although data for hospitalised and out-patients should be reported separately, this division has not always been done. Wide variations in bacterial resistance can be observed in different geographical areas and, even in the same area, can depend on the material studied. In addition, recording of data for repeat samples from the same patient may influence records of resistance levels (Huovinen 1986).

1.8.1 Developed countries

Urinary isolates

In the 1970's resistance to trimethoprim in urinary isolates in out-patients rarely exceeded 10% (Fleming *et al* 1972; Grüneberg 1976; Grey *et al* 1979; Dornbusch and Toivanen 1981; Hamilton-Miller *et al* 1981; Towner and Wise 1983; Mayer *et al* 1985). In the 1980's reports showed an increase in trimethoprim resistance, some centres reaching up to 20% (Huovinen *et al* 1982; Brumfitt *et al* 1983; Maskell 1983; Huovinen 1984; Mayer *et al* 1985; Kraft *et al* 1985; Huovinen and Toivanen 1986; Towner and Slack 1986; Heikkilä *et al* 1990a; Harnett 1992a). Increases in the levels of resistance to trimethoprim have levelled off at some centres in the 1980's and at some centres have even decreased (Huovinen *et al* 1985b; Amyes *et al* 1986b; Towner and Slack 1986; Heikkilä *et al* 1990b).

At Turku city hospital where trimethoprim use has been higher than in other parts of Finland (Huovinen and Toivanen 1980), resistance in *E. coli* isolated from the urinary infections of in-patients increased from 10-30% in the 1970's, and then increased further to 41% in 1984 (Huovinen *et al* 1986). Resistance has remained at a plateau of 40% from 1984-1988 (Heikkilä *et al* 1990b). Tsakris *et al* (1991) showed that trimethoprim resistance in Enterobacteria isolated from urine specimens of in-patients from Greek Hospitals was 30%. Resistance levels were generally shown to be higher in *Proteus* spp. and *Klebsiella* spp. than in *E. coli* (Huovinen and Toivanen 1980; Hamilton-Miller *et al* 1981; Towner and Wise 1983). Resistance levels in hospitalised

patients were usually double those of out-patients (Grüneberg 1976; Huovinen and Toivanen 1980; Brumfitt *et al* 1983; Towner *et al* 1983; Maskell 1983). The figures for resistance in Gram-negative bacilli from various infections isolated from in- and out-patients from Saint-Joseph Hospital, Paris from 1972 to 1979 showed a steady increase in resistance from 15% to around 25% (Acar and Goldstein 1982).

Enteric pathogens

Trimethoprim resistance among *Shigella* spp. has provided one of the most illustrative examples of the spread of trimethoprim resistance. In the Netherlands, resistance in *Shigella flexneri* rose from 7.6% in 1984 to above 25% in 1986 and has remained at that level from 1986 to 1989. In *Shigella sonnei*, the incidence of resistance rose steadily from 16% in 1984 to 46% in 1989. Over the same study period, resistance in *Shigella boydii* and *Shigella dysenteriae* isolates was 20% and 9.2% respectively (Voogd *et al* 1992). Harnett (1992b) showed that the levels of resistance in Canadian isolates of *S. flexneri, S. sonnei, S. dysenteriae* and *S. boydii* were 39.4%, 37.6%, 29.2% and 26.7% respectively. In a Spanish survey, 89% of *S. sonnei* isolates were resistant to trimethoprim (Palenque *et al* 1983). Resistance to *Salmonella* spp. is considerably lower (<10%) than in *E. coli* and *Shigella* spp. (Palenque *et al* 1983; Ward *et al* 1990). Resistance to trimethoprim in enteropathogenic *E. coli* isolated in the UK was 6% (Gross *et al* 1982).

1.8.2 Developing countries

Urinary isolates

The incidence of resistance to trimethoprim in developing countries is considerably higher than in developed countries despite a lower per capita antimicrobial use (Col and O'Connor 1987). There are little published data for the incidence of trimethoprim resistance in the 1970's in developing countries, however figures from the 1980's have shown that resistance levels have far exceeded those of developed countries. A study by Young *et al* (1986b) showed that the incidence of resistance to trimethoprim in South India in *Enterobacteriaceae* isolated from infected urines was 64%. Levels of resistance in Nigeria were similar at 63% (Lamikanra and Ndep 1989). In South Africa resistance in *Enterobacteriaceae* isolated from urine specimens at three hospitals was 38%, 53% and 60% (Wylie and Koornhof 1989). In Taiwan 34%, 30% and 42% of urinary isolates were resistant to trimethoprim in 1987, 1988 and 1989 respectively (Chang *et al* 1992).

Resistance to trimethoprim in *E. coli* from predominantly urine isolates in Chile, Thailand, Honduras, and Costa Rica was 44%, 40%, 38% and 48% respectively (Murray *et al* 1985). Amyes and Young (1987) showed that the incidence of trimethoprim resistance in *Enterobacteriaceae* isolated from all types of Gram-negative infection in Tanzania was 36%. A more recent study in Tanzania by Gillespie *et al* (1992) showed that trimethoprim resistance in *E. coli* and *Proteus* spp. was 64.5% and 61.5% respectively. In a similar study in Chile, Urbina *et al* (1989) reported that resistance to trimethoprim in clinical isolates of *E. coli* and *Klebsiella pneumoniae* was 25% and 41% respectively.

Enteric pathogens

Murray *et al* (1985) reported that the incidence of trimethoprim resistance in *Shigella* spp. in Brazil and Bangkok was 22.8% and 24% respectively. In Chile resistance in *Shigella* spp. was 37% (Urbina *et al* 1989). Reports from Bangladesh showed that from 1983 to 1990 the incidence of trimethoprim resistance in *Shigella dysenteriae* 1 isolates was >70%, however resistance in non-*S. dysenteriae* 1 isolates rose steadily from <2% in 1983 to 38% in 1990 (Bennish *et al* 1992). A study of resistance in *Shigella* spp. isolated from Finnish travellers returning from developing countries showed that resistance to trimethoprim rose steadily from 3% in 1975-83 to 42% in 1988 (Heikkilä *et al* 1990c).

The incidence of resistance in *Salmonella* spp. at many centres appears to be lower than in *Shigella* spp. Chugh (1985) reported that in *Shigella* spp., diarrheogenic *E. coli* and *Salmonella* spp. isolated in Kuwait, 43%, 59% and 15% of these isolates were resistant to trimethoprim respectively, while in Bangkok, 16% of *Salmonella* spp. were resistant. In Brazil however, resistance to *Salmonella* spp. and enteropathogenic *E. coli* was 76% and 50% respectively (Murray *et al* 1985). In Tanzania, resistance in *Salmonella* spp. was 53% (Gillespie *et al* 1983). Lamikanra *et al* (1990) reported that 38% of Nigerian isolates of diarrheogenic *E. coli* were trimethoprim-resistant. Transferable plasmids conferring trimethoprim resistance have been isolated from *Vibrio cholerae* in Tanzania (Young and Amyes 1986b) and in South India (Jesudason and John 1990).

1.9 Farming environment: a reservoir of resistance

The farming environment has been suggested as being another potential reservoir of antimicrobial resistance determinants. It has been reported that half the antibiotics produced in the United States are used in animal practice, either as therapeutic agents or as supplements in animal feeds (Levy 1984). It was thought that the development of resistance has been particularly encouraged by the use of antimicrobials at sub-therapeutic concentrations as supplements in animal feeds. These supplements were aimed at: infection prophylaxis, decreasing feed requirement and increasing the rate of weight gain (DuPont and Steele 1987). The Swann Report (Swann, 1969) recommended the restriction of the use of clinical antibiotics in animal feeds in the UK in order to reduce the high incidence of resistance associated with rearing livestock.

Trimethoprim-resistant *E. coli* have been isolated in 16% and 17% of porcine isolates in Sweden (Jansson *et al* 1992) and Scotland (Amyes 1987) respectively. Mee and Nikoletti (1983) showed that human and porcine isolates from Perth, Australia, shared a number of identical *E. coli* biotypes and both groups contained transferable R-factors of the same incompatibility group. Identical plasmids and resistant DHFRs have been shown to occur in both clinical and veterinary isolates (Amyes 1987; Jansson *et al* 1992). Linton *et al* (1972) reported that the incidence of faecal carriage of resistant organisms was significantly higher in people whose occupation involved contact with farm animals (63%) than in the control population (29%). These data suggest that conditions of modern stock breeding create large interconnecting bacterial populations with the potential for the development and spread of resistant organisms (Jansson *et al* 1993). A study conducted in Northern India by Singh *et al* (1992a) showed that the incidence of resistance was higher in human clinical isolates than in veterinary isolates and it was suggested that these differences occurred as a result of the infrequent use of antimicrobials in animal husbandry in India.

1.10 Mechanisms of spread of trimethoprim resistance genes

1.10.1 Clonal spread

Increases in the incidence of resistance as a result of the spread of a single resistant clone have been well documented. Wingard *et al* (1993) showed that in nursing home patients, 68% of acquired infections caused by trimethoprim-resistant Gram-negative bacilli occurred as the result of cross-colonisation. Since high levels of cross-colonisation were correlated with a lower functional level in the patients, it has been suggested that since these patients require a considerably greater amount of attention from health care workers, they are at higher risk of exposure to cross-colonisation. Other vehicles of spread have been identified outside the clinical environment. Food contaminated with resistant organisms has resulted in outbreaks of infection caused by resistant strains (Voogd *et al* 1992). As a result of human and animal faecal contamination, drinking water in many developing countries and recreational waters in developed countries have been shown to contain resistant *Enterobacteriaceae* and are thought to enhance the spread of resistance genes into food, animal and human populations (Young 1993).

1.10.2 Conjugation

The presence of R-factors conferring high levels of resistance to trimethoprim was first identified by Fleming *et al* (1972). It was shown that these plasmids were capable of transferring trimethoprim resistance *in vitro* to plasmid free strains of *E. coli* and *Salmonella typhimurium.* Studies from the early 1970's showed that high-level resistance (MIC≥1000mg/l) which is indicative of the production of an additional resistant DHFR occurred in less than 12% of trimethoprim-resistant isolates (Jobanputra and Datta 1974; Brumfitt *et al* 1980; Hamilton-Miller *et al* 1981). In most of these isolates high-level resistance was transferable (Jobanputra and Datta 1974; Grey *et al* 1979). While the incidence of low and intermediate levels of resistance (MIC<1000mg/l) has not increased significantly over time, by the late 1970's and early 1980's, the incidence of high-level resistance to trimethoprim had increased and accounted for between 60% and 86% of resistant isolates (Towner *et al* 1980; Brumfitt *et al* 1980; Hamilton-Miller *et al* 1981; Murray *et al* 1982; Kraft *et al* 1983; Kraft *et al* 1984; Towner and Wise 1983; Kraft *et al* 1985; Huovinen *et al* 1985b). The transfer of high-level resistance occurred in 59%-64% of these isolates (Grey *et al* 1979; Kraft *et al* 1982; Kraft *et al* 1983; Kraft *et al* 1985). This suggests

that the increase in the incidence of resistance to trimethoprim has occurred as the result of the subsequent spread of plasmid mediated DHFRs. Studies conducted in the 1980's and early 1990's on clinical isolates of *Enterobacteriaceae* from centres around the world have shown that high-level resistance to trimethoprim still occurs in the majority (57%-100%) of isolates (Chugh 1985; Young *et al* 1986b; Urbina *et al* 1989; Wylie and Koornhof 1989; Lamikanra and Ndep 1989; Lamikanra *et al* 1990; Tsakris *et al* 1991; Harnett 1992a). Resistance was transferable in 36% to 76% of these isolates.

Rudy and Murray (1984) demonstrated the presence of a transferable 'epidemic' trimethoprim resistance plasmid in 12/20 faecal isolates from Mexico. The plasmids were isolated from *E. coli* strains which represented five different serotypes, suggesting that horizontal transfer by conjugation has enhanced the spread of this plasmid. The phenomenon of different host strains harbouring identical plasmids has also been demonstrated in clinical isolates of *Enterobacteriaceae* (Mayer *et al* 1985) and between isolates from human and animal populations (Campbell *et al* 1986).

Whilst the prevalence studies have usually shown a high incidence of transferability to laboratory recipient strains, the relevance of this to transfer rates *in vivo* is not clear (Lacey 1975). It is probable that the transfer of R-plasmids amongst members of the commensal flora in the gut is a rare event and is therefore difficult to study *in vivo*. Experimental support for the rare nature of this transfer event was produced by Anderson *et al* (1973) who fed four normal subjects with about 10^{11} potential recipient *E. coli* then a few hours later with a similar number of donor *E. coli*, and looked for plasmid transfer. On the 16 occasions these experiments were performed, no plasmid transfer occurred. It was suggested that *Bacteroides* spp. in the gut flora were interfering with plasmid transfer. However, administration of an appropriate antibiotic resulted in transfer at high frequency (Anderson *et al* 1973; Anderson 1974). Despite the fact there is only one reliable report of *in vivo* plasmid transfer without selection pressure (Petrocheilou *et al* 1976), the potential for resistance transfer without antimicrobial selection pressure should not be eliminated.

1.10.3 Transformation and transduction

Transformation and transduction have been suggested as possible mechanisms by which resistance genes can spread between different species of bacteria (Levy 1982). Transformation has been demonstrated to occur naturally in species such as

Streptococcus pneumoniae, *Haemophilius influenzae* and *Neisseria meningitidis* (Dowson *et al* 1989; Powell and Livermore 1990; Spratt *et al* 1989). The importance of this type of transfer has been demonstrated by the occurrence of 'mosaic' genes which are interspersed with related fragments of foreign DNA and encode penicillin binding proteins which have a reduced affinity to β-lactam antibiotics. In *N. meningitidis* the transformed DNA fragments were found to be homologous to those of *Neisseria flavescens* (Spratt *et al* 1989), however the origins of the DNA fragments in *S. pneumoniae* have not been traced (Dowson *et al* 1989). It is thought that this mechanism of gene exchange may be of no importance in *Enterobacteriaceae* since they require considerable laboratory manipulation in order to be transformed (Murray 1991).

The best described examples of transduction are antibiotic resistance plasmids of *Staphylococcus aureus* that can be transduced by phage from strain to strain (Novick and Morse 1967). In theory, phage can transduce resistance genes from the chromosome of one species to another, or the resistance gene can originate from the phage itself. Since T4 phage encode a DHFR that forms both part of its structural and soluble portions (Matthews 1971) it was though that phage may be the source of trimethoprim-resistant DHFRs (Fling and Elwell 1980). However the T4 enzyme was shown to be very sensitive to inhibition by folate analogues (Mathews 1971). Other phage types however should not be ruled out as potential sources of resistant DHFRs.

1.10.4 Transposable elements

As the structures involved in genetic recombination and rearrangement of resistance genes are elucidated, their increasing importance in the role played in the emergence and spread of resistance genes is being recognised. A number of different mechanisms have been identified: these include insertion sequences (IS-elements), various classes of transposons, and the more recently identified element which is capable of site-specific insertion, the integron (Stokes and Hall 1989).

Insertion sequences

Insertion sequences are a special class of transposable elements ranging between 0.7-2kb and contain perfect or nearly perfect inverted terminal repeat sequences. The central region flanked by the terminal repeats contains at least one open reading frame, presumably for the transposase protein (Carlos and Miller 1980). One evolutionary implication derives from their ability to cause insertion mutations. These

mutations have the potential to inactivate genes, or if inserted into a suitable site, can activate cryptic genes or increase the expression of nearby genes (Sawyer *et al* 1987). Neis *et al* (1986) demonstrated that the presence of multiple copies of the IS-element IS*160* in the resistance plasmid pBP16 resulted in various types of rearrangements of the plasmid and its derivatives. These included inversion, deletion, replicon fusion and transposition. Since IS-elements are common in R-factors, it is likely that they are one of the main reasons for plasmid instability, and that they are involved in a major way in plasmid evolution.

Transposable elements

A pair of insertion sequences flanking a central sequence can transpose as a unit and give rise to what are called class I transposons. Such composite transposons containing antibiotic-resistance genes such as Tn*5*, Tn*9* and Tn*10*, are well documented (Carlos and Miller 1980). Class II or complex transposons are flanked by short inverted repeats of 30-40bp, but do not have IS-elements at their ends. In these elements the central sequence contains transposition genes and frequently antimicrobial resistance genes. Well documented examples of these are Tn*3* which carries ampicillin resistance and Tn*21* which has been shown to carry resistance to sulphonamides, trimethoprim, mercuric ions and streptomycin (Schmitt 1986). Unlike IS-elements and class I transposons which replicate conservatively, class II transposons are though to transpose via the formation of a cointegrate intermediate and replication of the transposon occurs during cointegrate formation (de la Cruz and Grinsted 1982). Transposons can also transpose into plasmids and remould their structure, and in general change the genetic capabilities of plasmids. By providing sites for replicon fusion they can also enable the transfer of non-conjugative plasmids during conjugation (Sawyer *et al* 1987). Several other structures bearing a complex array of transposition genes have been identified and are unrelated to the before mentioned groups. One such example of these is Tn*7* (Barth *et al* 1976).

Integrons

Recently a new type of element, the integron has been described in plasmids and transposons of Gram-negative bacteria, which may have great importance for the development of multiple resistance on a single mobile genetic element. Integrons are characterised by conserved 5' and 3' ends which flank a variable DNA segment. A gene coding for an enzyme, integrase, is located at the 5' end, and mediates site-specific integration of defined gene cassettes within the variable region of the

integron. The 3' end encodes two open reading frames of unknown function and a gene for sulphonamide resistance (Stokes and Hall 1989).

The cassettes which are integrated into the variable region are flanked by a highly conserved seven base element GTTRRRY (R=G or A; Y=C or T) at the 5' end, and a sequence of nucleotides known as the 59-base element located 3' to the structural gene (Hall *et al* 1991). Only 13 conserved bases of the proposed 59-base element have been estimated to be necessary for recombination to occur (Zühlsdorf and Weidemann 1993). Cassettes present within integrons can be excised through the action of integrase and have been identified as circular molecules within the cytoplasm of cells (Collis and Hall 1992). They are however, unable to replicate autonomously and must, therefore, be incorporated back into an integron structure to ensure maintenance and survival. A number of different resistance-gene cassettes have been found inserted within integrons: they include β-lactamase genes such as OXA and PSE, aminoglycoside modifying enzymes, chloramphenicol resistance genes (Bissonnette and Roy 1992) and trimethoprim-resistant DHFRs Ia, II, V, VII, X and XII (Huovinen *et al* 1995). The integration system described in Tn*21* has been shown to occur coupled with a number of other previously described Tn*21*-like structures. It has also been shown to occur without the presence of these structures, suggesting that the integrase system has evolved separately from these structures (Zülsdorf and Weidemann 1992). Integrons have been shown to be common in recently isolated clinical bacteria and have been shown to possess a number of different combinations of resistance genes (Lévesque *et al* 1995).

1.11 Aims of this thesis

Between January and March 1992, a survey was conducted by Shanahan *et al* (1993) to determine the incidence of antimicrobial resistance in the commensal faecal flora of healthy individuals resident in South Africa. Seventy-four percent of the population harboured trimethoprim-resistant organisms, from which 357 resistant strains were isolated. The aims of this thesis were to:

- Identify the trimethoprim-resistant isolates and characterise the resistance determinants associated with trimethoprim resistance.

- Determine whether trimethoprim resistance in these isolates was transferable to an *E. coli* recipient strain, and to determine which resistance determinants co-transferred with trimethoprim.

- Determine the MICs of trimethoprim for the wild-type isolates and their transconjugants.

- Develop oligonucleotide probes capable of identifying and discriminating between closely related DHFR genes.

- Determine the incidence of known trimethoprim-resistant DHFR genes in these isolates and to identify their potential mechanisms of spread.

- Identify and characterise by nucleotide sequence and biochemical properties any novel DHFR genes in the population.

2.0 MATERIALS AND METHODS

"Everything is vague to a matter of degree, you do not realise until you have tried to make it precise. "

Bertrand Russell

2.1 Chemical reagents

All reagents were supplied by Sigma chemicals (Dorset, UK) unless otherwise stated.

2.2 Bacteria strains and plasmids

The bacterial strains and plasmids used are listed in table 2.1.

Table 2.1. Strains of *E. coli* and plasmids.

Strain/Plasmid	Characteristics	Source/Reference
Strains		
J62-1	*his pro trp* NA^r	(Bachmann 1972)
J62-2	*his pro trp* Rif^r	(Bachmann 1972)
K802	*supE hsdR gal metB* Rif^r	(Wood 1966)
C600	*supE44 hsdR? thi-1 thr-1 leuB6 lacY1 tonA21*	(Bachmann 1972)
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	(Brown 1991)
Plasmids		
Transfer factor X^+		(Young *et al* 1986b)
pUC18	Ap^r	(Yanisch-Perron *et al* 1985)

2.3 Collection of trimethoprim-resistant isolates

Between January and March 1992, a survey was conducted by Shanahan *et al* (1993) to determine the incidence of antimicrobial resistance in the commensal faecal flora of healthy individuals resident in South Africa. The study examined eight separate population groups from both rural and urban areas. In the urban area the four groups comprised infants attending either a childminder or a crèche in SOWETO (0-5 years), urban children (6-11 years) and urban teenagers (12-19 years) attending a school in Kagiso, a town on the West Rand, and urban adults (>19 years) who resided in SOWETO. The rural population was composed of infants attending a 'well baby' clinic at Middelplaas in the KaNgwane district, rural children and rural teenagers attending a school at Hekpoort in the Magaliesburg district and lastly adults resident in KaNgwane district. A map showing the regions from which the specimens were collected from appears in figure 2.1.

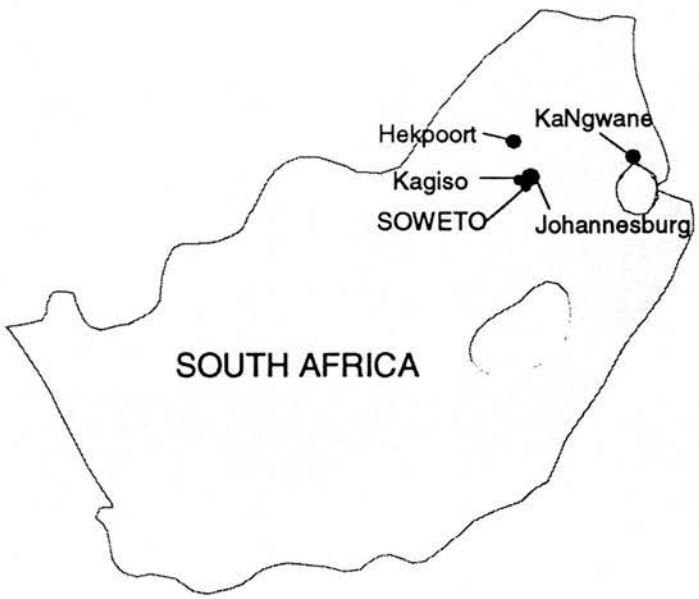


Figure 2.1. Map showing the study area from which specimens were collected.

A total of 361 specimens were obtained. Following the protocol established for such studies (Amyes *et al* 1992a), each specimen was plated onto modified Difco Mueller Hinton Agar (Detroit, USA) plates containing 10mg/L trimethoprim as described by Amyes and Gould (1984). Seventy five percent of the specimens contained trimethoprim-resistant organisms, from which 357 resistant strains were isolated. Table 2.2 shows the number of trimethoprim-resistant organisms isolated from each population group.

Table 2.2. Trimethoprim-resistant organisms isolated from each population group (Shanahan *et al* 1993).

Population	Region	No. of individuals	No. carrying resistant strains	Total resistant strains isolated
Urban infants	SOWETO	45	42	56
Urban children	Kagiso	42	34	39
Urban teenagers	Kagiso	47	23	26
Urban adults	SOWETO	44	35	43
Rural infants	KaNgwane	50	40	53
Rural children	Hekpoort	47	23	26
Rural teenagers	Hekpoort	36	34	44
Rural adults	KaNgwane	50	41	70
Total		361	272	357

2.4 Bacterial identification

Bacterial isolates were identified with standard biochemical tests (Collee *et al* 1989). The identification of 25 isolates which displayed unusual biochemical profiles was confirmed with the API20E (Bio Meriéux, France) identification kit.

2.5 Disc susceptibility testing

Antibiotic sensitivity tests were performed by the Kirby-Bauer disk diffusion method according to the guidelines set by the National Committee for Clinical Laboratory Standards (1990) on Iso-sensitest agar (Oxoid, UK). Disks (Mast Merseyside, UK) contained ampicillin (10μg), trimethoprim (5μg), piperacillin (100μg), amikacin (30μg), tetracycline (30μg), gentamicin (10μg), tobramycin (10μg), ceftazidime (30μg), cephazolin (30μg), cefotaxime (30μg), netilmicin (30μg), ciprofloxacin (5μg), sulfisoxazole (300μg), nalidixic acid (30μg), nitrofurantoin (300μg), streptomycin (10μg), spectinomycin (100μg), (UpJohn, USA) and chloramphenicol (30μg), (Abtek Biologicals, UK). The plates were incubated at 37°C for 16h.

2.6 Determination of minimum inhibitory concentrations

The MIC of trimethoprim for each isolate was determined by the agar dilution method according to the guidelines set by the National Committee for Clinical Laboratory Standards (1990). Davis Mingioli minimal medium (Davis and Mingioli 1950) supplemented with glucose 2.8g/L and appropriate concentrations of trimethoprim (Wellcome Medical Division, Crewe, UK) was used. Isolates that did not grow on this medium were tested on Davis Mingioli minimal medium containing thymidine (25mg/l) and appropriate concentrations of trimethoprim (Amyes and Smith 1974c). Organisms that failed to grow on this supplemented medium were re-tested on Iso-sensitest Agar (Oxoid, U.K.) with appropriate concentrations of trimethoprim. The Davis Mingioli minimal medium and the Iso-sensitest plates were incubated at 37°C for 40h and 16h, respectively.

2.7 Conjugation

Conjugation experiments were performed in liquid media by the method of Amyes and Gould (1984). *Escherichia coli* K-12 strain J62-2 (*his, trp, pro,* Rif^r) was used as the recipient. Isolates resistant to rifampicin were mated with *E. coli* K-12 strain J62-1 (*his, trp, pro,* NA^r). The selective medium used was Davis Mingioli minimal medium (Davis and Mingioli 1950) with glucose 2.8g/L, trimethoprim 10mg/L, rifampicin 25mg/L (Gruppo Lepetit, Milan, Italy) or nalidixic acid 20mg/L (Sanofi Winthrop, Guildford, UK), supplemented with L-histidine 50mg/L, L-proline 50mg/L and L-tryptophan 50mg/L. Colonies were purified on selective medium and their auxotrophic requirements checked on Davis Mingioli minimal agar supplemented with glucose 2.8 g/L. For the isolates which did not transfer trimethoprim resistance to the recipient strain, the mating was repeated with the inclusion of transfer factor X^+ as described by Young *et al* (1986b).

Filter matings were performed with a rifampicin resistant mutant of *E. coli* K802 (Wood 1966). Matings were prepared by passing 1ml of a 1:10 mixture of an overnight culture of donor and recipient strain respectively through sterile 13mm cellulose nitrate filters (pore size 0.45μm). The filters were placed onto the surface of Iso-sensitest agar and incubated overnight at 22°C or 37°C. The filters were placed in 10ml of Davis Mingioli minimal medium and shaken vigorously. A 100μl of cell suspension was plated out onto Iso-sensitest Agar with trimethoprim 10mg/L and

rifampicin 25mg/L. The auxotrophic requirements of the transconjugants were confirmed on Davis Mingioli minimal agar supplemented with glucose 2.8 g/L.

2.8 Transformation

Plasmids from transconjugants which contained two or more plasmids were transformed into *E. coli* K12 C600 in order to isolate the plasmid which conferred trimethoprim resistance. Transformation experiments were done with a standard $CaCl_2$ transformation protocol (Maniatis *et al* 1982).

2.9 Plasmid isolation and restriction analysis

Plasmids were isolated according to the method of Birnboim and Doly (1979) with the following modifications: The volumes of culture, lysozyme solution, lysing solution and high salt solution were doubled. Protein in the cleared lysate was extracted with phenol/chloroform (1:1). The DNA was precipitated with one volume of isopropanol and spun in an Eppendorf 5415 microcentrifuge at 15 000xg for 10 min at room temperature. The pellet was washed with 70% ethanol and dried. The plasmid DNA was resuspended in 30µl distilled water with 20µg/ml RNase. The DNA was digested for 2h at 37°C with 10U of *Eco*RI restriction endonuclease (Boehringer Mannheim, Germany) according to the manufacturer's instructions. The digested DNA was analysed by electrophoresis on a 0.8% agarose gel run overnight at 3V/cm. Plasmid size was determined on agarose gels by comparing the electrophoretic mobility of the fragments of restricted plasmids with restricted λ DNA fragments of known molecular weight.

2.10 DNA hybridisation protocols

2.10.1 Probe construction

Probes to distinguish between the type Ib and V DHFRs were used as described by Young *et al* (1994). The region used is very heterogeneous between these two genes. Considerable heterogeneity occurs in the same region throughout all resistant DHFRs and this region was selected for the construction of 30-bp oligonucleotide probes for the type Ia, Ib, IIIa, V, VI, VII, VIII, IX, X, and XII DHFR genes. Figure 2.2 shows the alignment of the amino acid sequence of these DHFR genes and the respective regions from which the oligonucleotide probes were derived. The nucleotide sequence

```
DHFR          1        10        20        30        40        50        59
Ia              MKLSLM-VALSKNGVIGNGPDIPW-SAKGEQLLFKAITYNQW-LLVGRKTFESMG-ALP-
Ib            MRTLKVSLI-AAKRKNGVIGCGPDIPW-SAKGEQLLFKALTYNQC-LLVGRKTFESMG-ALP-
V               MKVSLM-AAKAKNGVIGCGPHIPW-SAKGEQLLFKALTYNQW-LLVGRKTYESMG-ALP-
VI              MKISLM-AAVSENGVIGSGLDIPW-HVQGEQLLFKAMTYNQW-LLVGRKTFDSMG-KLP-
VII             MKISLI-SATSENGVIGNGPDIPW-SAKGEQLLFKALTYNQW-LLVGRKTFDSMG-VLP-
IIIa            MLISLI-AALAHNNLIGKDNLIPW-HLPADLRHFKAVTLGKP-VVMGRRTFESIGRPLP-
VIII          MIELHAI-LAATANGCIGKDNALPWPPLKGDLARFKKLTMGKV-VIMGRKTFESLPVKLE-
IX              MASLNMI-VAVNKTGGIGFENQIPW-HEPEDLKHFKAVTMNSV-LIMGRKTFASLPKVLP-
X               MNISLIFANELITRAFGNQGKLPW-QFIKEDMQFFQKTTENSVVVMGLNTWRSLPKMKKL
XII       MNSESVRIYLV-AAMGANRVIGNGPNIPW-KIPGEQKIFRRLTEGKV-VVMGRKTFESIGKPLP-
E. coli         MISLI-AALAVDRVIGMENAMPW-NLPADLAWFKRNTLNKP-VIMGRHTWESIGRPLP-

DHFR  60        70        80        90        100       110       120
Ia      NRKYAVVTRSSFTSDNENVLIFPSIKDALTN-LKKITD-----HVIVSGGGEIYKSLIDQVDTLHI
Ib      NRKYAVVTRSGWTSNDDNVVVFQSIEEAMDR-LAEFTG-----HVIVSGGGEIYRETLPMASTLHL
V       NRKYAVVTRSAWTADNDNVIVFPSIEEAMYG-LAELTD-----HVIVSGGGEIYRETLPMASTLHI
VI      NRKYAVVTRSKIISNDPDVVYFASVESALAY-LNNATA-----HIFVSGGGEIYKALIDEADVIHL
VII     NRKYAVVSRKGISSSNENVLVFPSIEIALQE-LSKITD-----HLYVSGGGQIYNSLIEKADLIHL
IIIa    GRRNVVVSRNPQWQAE-GVEVAPSLDAALA--L--LTDCE---EAMIIGGGQLYAEALPRADRLYL
VIII    GRTCIVMTRQALELP--GVRDANGAIFVNNVSDAMRFAQEE------SVGDVAYVIGGAEIFKRLA
IX      GRLHVVVSKTVPPTQNTDQVVTVSTYQIAVRTASLLVDLPEYSQIFVIGGKSAYENLAAYVDKLYL
X       GRDFIVIS-STIT-EHEVLNNNIQIFKSFESFLEAFRDTTK--PINVIGGVGLLSEAIEHASTVYM
XII     NRHTLVISRQANYRATGCVVV-STLSHAIALASELGN------ELYVAGGAEIYTLALPHAHGVFL
E.coli  GRKNIILS-SQPGTD-DRVTWVKSVDEAIAACGDVP-------EIMVIGGGRVYEQFLPKAQKLYL

DHFR      130       140       150       160       170       180       190
Ia      STIDIEPEGDVYFPEI-PSNFRPVFT-QDFASN---INYSYQIWQKG
Ib      STIDIEPEGDVFFPSI-PNTFEVVFE-QHFTSN---INYCYQIWKKG
V       STIDIEPEGDVFFPNI-PNTFEVVFE-QHFSSN---INYCYQIWQKG
VI      SVIHKHISGDVFFPPV-PQGFKQTFE-QSFSSN---IDYTVQIWAKG
VII     STVHVEVEGDINFPKI-PENFNLVFE-QFFLSN---INYTYQIWKKG
IIIa    TYIDAQLNGDTHFPDYLSLGWQELERSTHPADDKNSYACEFVTLSRQR
VIII    LMITQIELTFVKRL-YEGDTYVDLAEMVKD-YEQNGMEEHDLHTYFTYRKKELTE
IX      TRVVQLNTQQDTELDLSLFKSWKLVSEVPTITGNKTKLIFQIWINPNPISEEPTC
X       SSIHMVKPVHADVYVPVELMNKLYSDFKYPENILWVGDPIDSVYSLSIDKFVRPASLVGVPNDINT
XII     SEVHQTFEGDAFFPMLNETEFELVSTETIQAVIPYTHSVYARRNG
E.coli  THIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERR
```

Figure 2.2. Alignment of the amino acid sequence of the types Ia, Ib, V, VI, VII, IIIa, VIII, IX, X, XII and the chromosomal DHFR from *E. coli* K12. The underlined region represents the heterogeneous region from which the DHFR oligonucleotide probes were selected.

Table 2.3. Oligonucleotides for DHFR gene probes.

DHFR	Oligonucleotide sequence	Origin/Reference
Ia	5'-CAAGTTTTACATCTGACAATGAGAACGTAT-3'	Nucleotide 429-459 (Fling and Richards 1983)
Ib	5'-GTTGGACATCAAATGATGACAATGTAGTTG-3'	Nucleotide 430-460 (Young *et al* 1994)
IIIa	5'-ATCCCCAATGGCAGGCCGAAGGGGTGGAGG-3'	Nucleotide 298-328 (Fling *et al* 1988)
VIII	5'-AAGCGCTGGAGCTTCCGGGTGTTCGTGACG-3'	Nucleotide 779-809 (Barg *et al* 1995)
V	5'-CCTGGACGGCCGATAATGACAACGTAATAG-3'	Nucleotide 1501-1531 (Sundström *et al* 1988)
VI	5'-CTAAAATTATCTCGAATGACCCTGATGTTG-3'	Nucleotide 528-558 (Wylie and Koornhof 1991)
VII	5'-GAATTTCAAGCTCAAATGAAAATGTATTAG-3'	Nucleotide 789-819 (Sundström *et al* 1993)
IX	5'-CAGTACCACCCACCCAGAACACTGATCAAG-3'	Nucleotide 924-954 (Jansson *et al* 1992)
X	5'-CAACTATCACAGAGCACGAAGTGCTCAACA-3'	Nucleotide 848-878 (Parsons *et al* 1991)
XII	5'-AAGCTAACTACCGCGCCACTGGCTGCGTAG-3'	Nucleotide 414-444 (Singh *et al* 1992b)

of the 30mer oligonucleotide probes for the DHFRs appear in Table 2.3. All of the oligonucleotide probes were tested for homology with other DNA sequences on the GenBank database. The probe for the integrase gene of Tn*7* was previously described (Heikkilä *et al* 1991). The probe for the integrase gene of Tn*21* was a 22mer oligonucleotide probe 5'-GTCAAGGTTCTGGACCAGTTGC-3' derived from the nucleotide sequence of the integrase gene of Tn*21* (Sundström *et al* 1993). The oligonucleotides were synthesised by Oswel DNA service (Edinburgh University). The oligonucleotide probes were labelled with the ECL Oligo labelling and detection kit (Amersham, UK). The type II gene probe consisted of a 275-bp *Eco*RI, *Sau*3AI intragenic fragment of pWZ820 (Zolg *et al* 1978) and was labelled with the ECL Randomprime labelling and detection kit (Amersham, UK). Table 2.4 shows the control strains that were used with the probes.

Table 2.4. Control plasmids used in hybridisation protocols.

Plasmid	DHFR	Reference
pFE506	*dhfr Ia*	(Fling and Richards 1983)
pUK163	*dhfr Ib*	(Young *et al* 1994)
R67	*dhfr IIa*	(Smith *et al* 1979)
R388	*dhfr IIb*	(Swift *et al*1981)
R751	*dhfr IIc*	(Flensburg and Steen 1986)
pAZ1	*dhfr IIIa*	(Fling*et al* 1988)
pBH600	*dhfr IIIb*	(Barg *et al* 1990)
pBH700	*dhfr IIIc/VIII*	(Barg *et al* 1990)
pUK1123	*dhfr IV*	(Young and Amyes 1986a)
pLMO20	*dhfr V*	(Sundström *et al* 1987)
pUK672	*dhfr VI*	(Wylie *et al* 1988)
pLMO27	*dhfr VII*	(Sundström *et al* 1987)
pCJO01	*dhfr IX*	(Jansson *et al* 1992)
pMAQ41	*dhfr X*	(Parsons *et al* 1991)
pBEM155	*dhfr XII*	(Singh *et al* 1992b)

2.10.2 DNA isolation

Plasmid DNA was isolated as described previously in 2.9. Total DNA was isolated by the following method. All manipulations were carried out at room temperature in 1.5ml micro centrifuge tubes unless otherwise stated. Cells were harvested from 0.5ml of overnight Luria broth (1% Difco Bacto-tryptone, 0.5% Difco yeast extract and 1% NaCl, pH7.0) culture and resuspended in 350µl of high Tris, EDTA (HTE) buffer (50mM Tris-Cl, pH8.0, 20mM EDTA). An equal volume of 2% *N*-lauroylsarcosine in HTE buffer was added and incubated at 55°C for 30 min to lyse the cells. The lysate was cooled to room temperature then extracted with one volume of phenol:chloroform (1:1, equilibrated with 0.5M Tris-Cl, pH8.0). One volume of isopropanol was added to the supernatant and the DNA was precipitated for 10 min prior to centrifugation. The DNA pellet was resuspended in 50µl of H_20 with RNase 20µg/ml.

2.10.3 Dot blotting procedure

The concentration of plasmid and total DNA samples were estimated by agarose gel electrophoresis. Samples were denatured by the addition of one volume of 1M NaOH. Approximately 20ng of plasmid DNA or 500ng of total DNA was spotted onto nitrocellulose membranes (DuPont Genescreen, Germany). The membranes were dried then baked at 80°C for two hours.

2.10.4 Hybridisation with oligonucleotide probes

Oligonucleotide ECL Oligo labelling and detection kit (Amersham, UK). The hybridisation was performed for two hours at 53°C according to the manufacturer's recommendations. Stringency washes were performed as described previously by Heikkilä *et al* (1991).

2.10.5 Hybridisation with gene probes

Gene probes were labelled and positive hybridisation results were detected by means of the ECL Randomprime labelling and detection kit (Amersham, UK). The blots were hybridised overnight at 62°C according to the manufacturer's recommendations with the following stringency washes: two 15 minute washes with 1x SSC and 0.1% SDS followed by two 15 minute washes with 0.05x SSC and 0.1% SDS at 62°C.

2.10.6 Southern Hybridisation

Plasmid and total DNA samples were run on 0.8% agarose gels and Southern blotted onto nitrocellulose membranes (Amersham, Hybond C-Extra) according to the method of Southern (1975). Total DNA that was restricted with *Eco*RI or *Bam*HI was run on 1% agarose gels before Southern blotting.

2.11 Enzyme preparation and assay

2.11.1 Preparation of dihydrofolate reductase.

Three litres of bacterial culture were grown overnight in Luria broth on a shaking platform at 37°C. The bacteria were harvested by centrifugation (3800g at 4°C for 10min). All further manipulations were carried out at 4°C. The bacterial pellet was washed with 100ml buffer A (50mM sodium phosphate buffer pH 7.4 containing 10mM β-mercaptoethanol and 1mM EDTA). The pellet was finally resuspended in 20ml ice cold buffer A. The suspension was sonicated for 3x45 sec with 1min intervals using a MSE Soniprep 150 then clarified by centrifugation at 40 000g for 45min. The resultant supernatant was the crude enzyme preparation. Nucleic acids were removed by the addition of 0.1 volume of 10% streptomycin sulphate followed by centrifugation at 20 000g for 30min. After centrifugation, ammonium sulphate was added to 50% saturation. The precipitate was removed by centrifugation at 20 000g for 30min. Ammonium sulphate was added to 80% saturation and the precipitate was removed by centrifugation at 20 000g for 30min. The pellet which contained most of the DHFR activity and hardly any NADPH oxidase activity was resuspended in 3ml buffer A and stored at -20°C.

2.11.2 Protein estimations

Estimation of protein concentrations in crude lysates were determined by the method of Waddel (1956). The absorbance of a diluted sample was measured at OD_{215} and OD_{225}. The protein concentration was then ascertained by comparing the difference between these readings with a previously prepared standard curve.

2.11.3 Sephadex gel-exclusion chromatography

The enzyme was separated at 4°C on a Sephadex G-75 column ($2cm^2$ cross sectional area x 90cm) by the method of Andrews (1964). Buffer A was washed through the column at a constant rate of 10ml per hour. Two millilitres of sample were applied to the column, and 2ml fractions were collected every 12min.

The column was calibrated by the method of Andrews (1964) with three proteins of known molecular weight: ovalbumin (M_r=45 000), chymotrypsinogen (M_r=25 500) and cytochrome C (M_r=12 384). Fifty milligrams of each protein were dissolved in

2ml buffer A and applied to the column. The protein concentration of each fraction was measured at OD_{280}. A standard curve was derived by plotting the eluted volume against the log of the molecular weight of the peak fractions of eluted protein.

2.11.4 Dihydrofolate reductase assays

Dihydrofolate reductase assays were performed according to the method of Osborn and Huennekens (1958). A Perkin Elmer lambda 2 spectrophotometer, with heated cuvette carriage (37°C) was used to measure the decrease in absorbance at OD_{340}. This is caused by the oxidation of NADPH to NADP in the reduction of dihydrofolate to tetrahydrofolate by the activity of DHFR. When both substrates are acted upon stoichiometrically, 52% of this combined fall in absorbance is a result of the reduction of dihydrofolate (Matthews *et al* 1963). With this qualification, the rate of enzyme activity is expressed in molar terms of dihydrofolate reduction to tetrahydrofolate.

The reaction was performed in 1ml quartz cuvettes containing 40mM sodium phosphate buffer pH 6.0, 10mM β-mercaptoethanol, 0.08mM NADPH, enzyme and distilled water to 950µl (Amyes and Smith 1978). A blank, which did not contain NADPH, and the sample were placed in the spectrophotometer and allowed to equilibrate for 3min. Any decrease in absorbance measured at this stage was taken as DHF-independent NADPH oxidase activity and was deducted from the final reading. Dihydrofolate was then added to both cuvettes and the decrease in absorbance was measured for 3min or until a zero reading was recorded.

2.11.5 Specific activity

The specific activity of the crude protein preparation was determined by dividing the activity of the sample (as determined in 2.11.4) by the total protein concentration.

2.12 Enzyme kinetics

The eluted fractions from the Sephadex G75 column which displayed maximum DHFR activity were pooled for further assay. The following assays were performed:

2.12.1 Drug inhibition

The dose of trimethoprim or methotrexate required to inhibit DHFR activity by 50% (ID_{50}) was assayed as above, in the presence of increasing concentrations of the two drugs.

2.12.2 Temperature sensitivity

To determine the exposure time at 45°C required to inhibit enzyme activity to 50% (TD_{50}), the enzyme was pre-treated at 45°C for time spans increasing from 0.5-12 min prior to each assay.

2.12.3 Michaelis constant

To determine the Michaelis constant (K_m) which is a measure of the enzyme's affinity for the substrate or, the substrate concentration at which the rate of reaction is half the maximum velocity, the enzyme was assayed in the presence of decreasing dihydrofolate concentrations. The results were then analysed by the method of Lineweaver and Burke: where the reciprocal of the substrate concentration $1/[S]$ is plotted against the rate of enzymic reaction $1/V$. When extrapolated, the interception on the abscissa gives the negative reciprocal of the K_m (Zubay 1993).

2.12.4 Trimethoprim inhibition constant

The inhibitor constant (K_i) is analogous to the enzyme substrate constant (K_m), and is defined as the dissociation constant of the enzyme-inhibitor complex (Zubay 1993). This was determined by repeating the assays for the K_m, in the presence of trimethoprim and then plotting the $1/[S]$ against $1/V$ as before. As a result of competitive inhibition of trimethoprim, the line intercepts the ordinate at the same point as the K_m, but the interception of the abscissa is nearer to the origin, as the reaction is slowed down as a result of trimethoprim inhibition. The negative reciprocal of the point of interception gives an apparent K_m, (the K_p) in the presence of the

inhibitor. The K_i is then determined from the following equation (Zubay 1993), where $[i]$ is the trimethoprim concentration:

$$K_i = \frac{[i]}{\frac{K_p}{K_m} - 1}$$

2.13 DNA cloning

Cloning procedures were performed as described by Maniatis *et al* (1982). Plasmid DNA from transconjugant and vector (pUC18) were isolated as described in 2.9. The DNA was restricted for two hours at 37°C with appropriate restriction enzymes and the recommended buffers as supplied by GibcoBRL (UK). Restricted DNA that was to be sub-cloned was separated on 0.8% agarose gels and the appropriate DNA fragments from the gel were excised and purified with the GeneClean kit (Bio101, La Jolla, USA). Restricted fragments were ligated with T4 DNA ligase (Gibco-BRL, UK) for 16 hours at 12°C. The ligated DNA was transformed into competent *E. coli* HB101 as prepared by a $CaCl_2$ transformation protocol described by Maniatis *et al* (1982). Putative transformants were selected on Iso-sensitest agar containing ampicillin 50mg/L and trimethoprim 20mg/L.

2.14 Nucleotide sequencing and analysis

The plasmid DNA containing the cloned fragment to be sequenced was isolated by a alkali lysis method described by Maniatis *et al* (1982). The DNA sample was further purified by agarose gel electrophoresis - the closed circular DNA was excised from the gel and purified with the GeneClean kit (Bio101, La Jolla, USA).

The template DNA was quantified by agarose gel electrophoresis. For each labelling reaction 3-5µg of plasmid DNA was denatured by the addition of 0.1 volumes of 2M NaOH and incubated for 30min at 37°C. The mixture was neutralised by adding 0.1 volumes of 3M sodium acetate (pH 4.8) and the DNA precipitated with two volumes of ethanol (-70°C, 15 min). The DNA pellet was washed with 70% ethanol and resuspended in 7µl of distilled water.

The sequencing reaction based on the chain termination method described by Sanger *et al* (1977), was performed using the SEQUENASE Version 2.0, DNA Sequencing kit (United States Biochemical, Cleveland, USA) and [^{35}S]-dATP (Amersham) label. The M13 -40 forward primer was supplied with the labelling kit, and the reverse primer 5'-GGAAACAGCTATGACCATG-3' was a gift from Dr H.-K. Young, Dundee University. Further primers were derived from the previously determined nucleotide sequences and were synthesised by Oswel DNA service (Edinburgh University). For each primer, two sequencing reactions were performed: for longer sequences, the labelling reaction time was extended to ten minutes and for determining sequences close to the primer the labelling reaction was performed for three minutes with the addition of 1µl Mn^{2+} buffer supplied with the kit.

The samples were electrophoresed for 2-5 hours at 60W with a Biorad sequencing apparatus. The gels were fixed for 30min and dried with a Biorad gel drier for two hours at 80°C under vacuum. Autoradiography was carried-out for 24 hours at room temperature with Amersham hyperfilm MP. The development of the film was kindly performed by the Edinburgh Royal Infirmary X-ray department.

Sequence comparisons were performed using the GCG sequence analysis software. Nucleotide sequences were compared with all bacteria sequences in the GenBank and EMBL databases using the FASTA search programme.

3.0 RESULTS

"The most savage controversies are those about matters as to which there is no good evidence either way."

Bertrand Russell

3.1 Identification of isolates

The majority of the 357 trimethoprim-resistant organisms which were isolated and identified were *Escherichia coli,* 297 (83.2%). The *Klebsiella* spp. were less frequent 46 (12.9%), followed by *Enterobacter* spp. 7 (2%), *Shigella sonnei* 3 (0.8%) and a single isolate each of *Proteus vulgaris*, *Kluyvera spp.*, *Citrobacter freundii* and *Pseudomonas aeruginosa.* The three *S. sonnei* isolates were isolated from volunteers who were asymptomatic.

3.2 Transfer of trimethoprim resistance

Trimethoprim resistance was transferable to an *E. coli* J62-2 (three plasmids from rifampicin resistant isolates were transferred into J62-1) recipient strain by conjugation in 184 (51.5%) of the 357 isolates. An X^+ factor was required to mobilise resistance in a further 12 (3.4%) isolates. Table 3.1 shows the proportion of isolates for each species that transferred trimethoprim resistance to the recipient strain.

Table 3.1. Number of transconjugants for each species.

Donor species	No. of isolates	Transconjugants(%)	X^+ Factor(%)
Escherichia coli	297	151 (50.8)	12 (3.4)
Klebsiella spp.	46	27 (58.7)	0
Enterobacter spp.	7	2 (28.6)	0
Shigella sonnei	3	3 (100)	0
Other genera†	4	1 (25)	0
Total	357	184 (51.5)	12 (3.4)

† *Kluyvera* spp. (n=1), *Proteus vulgaris* (n=1), *Pseudomonas aeruginosa* (n=1) and *Citrobacter freundii* (n=1).

The proportion of *E. coli* and *Klebsiella* spp. isolates that could transfer resistance by conjugation was similar (between 50-60%). In contrast all three of the *S. sonnei* isolates and only 29% of the *Enterobacter* spp. isolates transferred resistance.

3.3 Antibiotic resistance associated with trimethoprim resistance

The trimethoprim-resistant isolates were tested, by the disk diffusion method, for resistance to the following antimicrobials: ampicillin, trimethoprim, piperacillin, amikacin, tetracycline, gentamicin, tobramycin, ceftazidime, cephazolin, cefotaxime, netilmicin and ciprofloxacin at the concentrations listed in the materials and methods. Resistance to two or more of these agents occurred in 94% of the isolates. Seventy-one percent of the isolates were resistant to three or more antimicrobials and 62% carried resistance to four or more agents. Only 12% of the isolates were resistant to five or more agents.

Tetracycline resistance was associated with trimethoprim resistance in 88% (317/357) of the isolates. Resistance to ampicillin occurred in 71.4% (255/357) and piperacillin in 66.7% (238/357) of the trimethoprim-resistant isolates. Piperacillin resistance was always associated with ampicillin resistance. Aminoglycoside resistance was uncommon as gentamicin resistance occurred in 3.4% (12/357), tobramycin in 3.4% (12/357), netilmicin in 1.4% (5/357) and amikacin in 0.3% (1/357) of the isolates. Resistance to first and third generation cephalosporins was also uncommon. Of the cephalosporins, resistance to cefazolin was the most common and occurred in 12.9% (46/357) of the isolates. Cefotaxime and ceftazidime resistance occurred in 1.7% (6/357) and 1.4% (5/357) of isolates. All the isolates were susceptible to ciprofloxacin. The combination of resistance to trimethoprim, tetracycline, ampicillin and piperacillin was the most prevalent and occurred in 48% of the isolates. Twenty-three percent of the isolates were resistant to a combination of trimethoprim and tetracycline. The antibiograms of the wild-type isolates are shown in table 3.2.

Table 3.2. Antibiograms of wild-type trimethoprim-resistant isolates.

Antibiogram	Number of isolates (%)
Tp	20 (5.6)
TpTc	82 (23)
TpAp	1 (0.3)
TpApPrl	15 (4.2)
TpTcAp	15 (4.2)
TpApCz	1 (0.3)
TpTcApCz	7 (2.0)
TpTcApPrl	170 (47.6)
TpTcApTn	1 (0.3)
TpApPrlCz	2 (0.6)
TpTcApPrlCz	28 (7.8)
TpTcApCzCtx	2 (0.6)
TpTcApPrlGm	1 (0.3)
TpTcApPrlAk	1 (0.3)
TpTcApPrlCzGmTn	6 (1.7)
TpTcApPrlCazGmTnNet	1 (0.3)
TpTcApPrlCzCazCtxGmTnNet	4 (1.1)

3.4 Resistance associated with transferable trimethoprim resistance

The transconjugants were tested by the disk diffusion method for resistance to ampicillin, piperacillin, amikacin, tetracycline, gentamicin, tobramycin, ceftazidime, cephazolin, cefotaxime, netilmicin and ciprofloxacin.

Resistance to antibiotics including trimethoprim occurred at the following frequencies: tetracycline 30% (59/196), ampicillin 55,6% (109/196), piperacillin 55.1% (108/196), gentamicin 2.6% (5/196), tobramycin 1.5% (3/196), ceftazidime 1.5% (3/196), cefotaxime 0.5% (1/196), cefazolin 0.5% (1/196). Resistance to netilmicin and amikacin did not transfer from donor to recipient. Figure 3.1 shows the percentage of transconjugants that were resistant to ampicillin, tetracycline, cefazolin and gentamicin in comparison to the incidence of resistance in the respective plasmid donors. Tetracycline, ampicillin, cefazolin, and gentamicin resistance co-transferred

with trimethoprim resistance in 34%, 77%, 14% and 83% of the isolates which were originally resistant to these antimicrobial agents respectively.

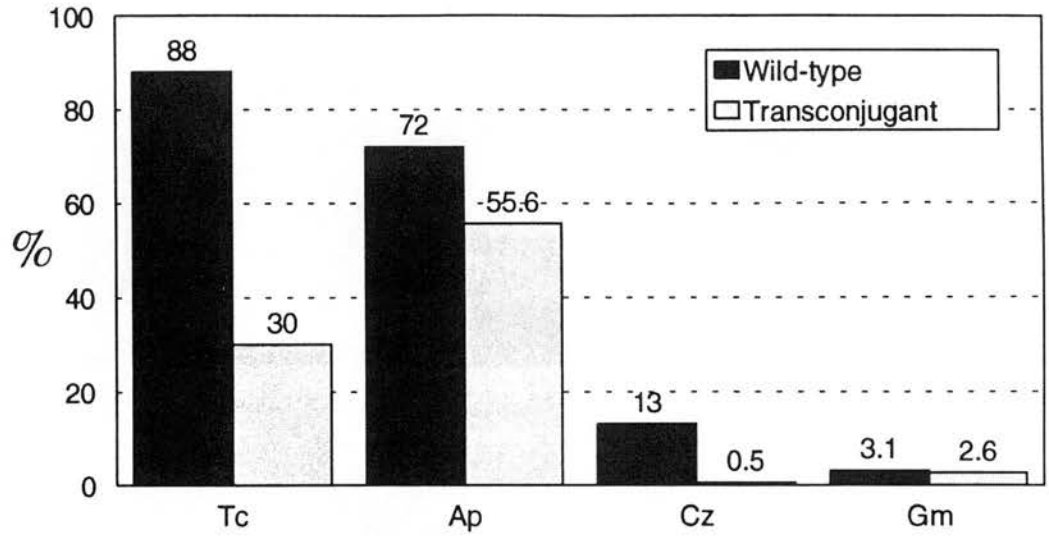


Figure 3.1. Antimicrobial resistance associated with trimethoprim resistance in the transconjugants and their wild-type plasmid donors.

To differentiate further between the different antibiogram types, the transconjugants were tested for resistance to sulfisoxazole, streptomycin, spectinomycin, chloramphenicol, nalidixic acid and nitrofurantoin. Resistance to antibiotics including trimethoprim occurred in the transconjugants at the following frequency: sulfisoxazole 90.8% (178/196), streptomycin 48% (94/196), spectinomycin 24% (47/196), chloramphenicol 11.7% (23/196) and nalidixic acid 1% (2/196). All the transconjugants were susceptible to nitrofurantoin.

Some resistance profiles occurred more frequently than others. Thirty-three percent (65/196) of the transconjugants were resistant to a combination of trimethoprim and sulfisoxazole. A combination of resistance to TpApPrlSxSm occurred in 19% (37/196) of the isolates, and combinations of TpTcApPrlSxSm and TpTcApPrlSxSmSpCm occurred in 8% (15/196) and 6% (12/196) of the isolates respectively. The antibiograms of the transconjugants appear in table 3.3.

Table 3.3. Antibiograms of the transconjugants.

Antibiogram	Number of transconjugants (%)
Tp	4 (2.0)
TpSx	65 (33.1)
TpTcSx	4 (2.0)
TpTcSp	1 (0.5)
TpSmSp	4 (2.0)
TpTcSxSp	6 (3.1)
TpTcGmSx	1 (0.5)
TpSmSpNA	2 (1.0)
TpApPrl	5 (2.6)
TpApPrlSx	10 (5.1)
TpApSxSm	1 (0.5)
TpTcApPrlCm	1 (0.5)
TpApPrlSxSm	37 (18.9)
TpApPrlSxSp	2 (1.0)
TpTcApPrlSxSm	15 (7.6)
TpTcApPrlSxSp	3 (1.5)
TpApPrlSxSmCm	1 (0.5)
TpTcApPrlSmSp	1 (0.5)
TpApPrlSxSmSp	4 (2.0)
TpTcApPrlSxSmSp	8 (4.1)
TpTcApPrlSxSmCm	4 (2.0)
TpApPrlSxSmSpCm	1 (0.5)
TpTcApPrlSxSmSpCm	12 (6.1)
TpTcApPrlGmSxSmCm	1 (0.5)
TpTcApPrlGmTnCazSxSmSpCm	1 (0.5)
TpTcApPrlGmTnCazSxSmSpCmCz	1 (0.5)
TpTcApPrlGmTnCazSxSmSpCmCtx	1 (0.5)

3.5 Minimum inhibitory concentrations of trimethoprim

The minimum inhibitory concentrations of trimethoprim in the wild-type isolates are shown in table 3.4 High-level resistance (MIC ≥1024 mg/l) occurred in 98.6% of the isolates. Seventy-nine percent of the isolates were resistant to more than 2048mg/l of trimethoprim. These figures suggest that the vast majority of trimethoprim resistance genes were of plasmid or transposon origin.

Table 3.4. Trimethoprim minimum inhibitory concentrations for the wild-type isolates.

	Trimethoprim MIC (mg/l)					
Species	>2048	2048	1024	512	256	16
E. coli (n=297)	233	60	2	2	0	0
Klebsiella spp (n=46)	41	4	0	0	0	1
Enterobacter spp (n=7)	5	0	0	0	2	0
S. sonnei (n=3)	2	1	0	0	0	0
Other genera† (n=4)	2	2	0	0	0	0
Total (n=357)	283	67	2	2	2	1

† *Proteus vulgaris* (n=1), *Kluyvera* spp. (n=1), *Citrobacter freundii* (n=1) and *Pseudomonas aeruginosa* (n=1).

Table 3.5 shows the MICs of trimethoprim in the transconjugants. The MICs of trimethoprim were on average lower in the transconjugants than in the wild-type isolates. High-level resistance (MIC ≥1024 mg/l) occurred in 85.7% of the transconjugants and only 46% of the isolates were resistant to more than 2048mg/l of trimethoprim. Differences between the trimethoprim MICs of the wild-type isolates and their transconjugants were most likely due to differences in the media used for susceptibility testing, (DM minimal medium and Iso-sensitest agar respectively) and differences in the DHFR specific activity of the isolates.

Table 3.5. Minimum inhibitory concentrations of trimethoprim for the transconjugants.

	Trimethoprim MIC (mg/l)					
Species of plasmid donor	>2048	2048	1024	512	256	128
E. coli (n=163)	65	46	24	17	10	1
Klebsiella spp. (n=27)	20	7	0	0	0	0
Enterobacter spp. (n=2)	2	0	0	0	0	0
S. sonnei (n=3)	2	0	1	0	0	0
Kluyvera spp. (n=1)	1	0	0	0	0	0
Total (n=196)	90	53	25	17	10	1

3.6 Plasmid analysis

Plasmids were extracted from the wild-type isolates and their corresponding transconjugants to determine whether trimethoprim resistance was plasmid mediated. All the transconjugants were demonstrated to have one or more plasmids of varying size. Twenty-four percent (48/196) of the transconjugants contained two or more plasmids. In order to obtain a restriction profile and antibiogram of the individual plasmid harbouring trimethoprim resistance, plasmids were isolated from these 48 transconjugants and transformed into *E. coli* (C600). Forty-one (85%) plasmids from these transconjugants could be transformed as single plasmids into an *E. coli* (C600) recipient. This is a significant proportion considering that most of these plasmids ranged between 50 and 100kb in size. Plasmid restriction profiles were obtained for all the transconjugants and transformants which had only one plasmid. A restriction profile of the individual plasmid which conferred trimethoprim resistance could not be determined from the remaining seven transconjugants bearing two or more plasmids.

A total of 107 different restriction profiles were recorded in the 189 transconjugants and transformants with single plasmids. The restriction profiles of plasmids which shared identical sized restriction fragments and elaborated identical antibiograms were defined as being the same plasmid. Table 3.6 lists the plasmids whose restriction

profile occurred in more than two transconjugants; the population group from which they came; the wild-type organism from which the plasmids were isolated and the resistance determinants carried by these plasmids. The restriction profiles of these plasmids are shown in figure 3.2.

Table 3.6. Common restriction profiles, the associated antibiograms, and the origin of the donor organism.

Plasmid	Number of isolates (%)	Population Group	Identity of Plasmid Donor	Resistance Markers
pUK2301	26 (13.8)	Urban infants (4) Urban children (6) Urban teenagers (1) Rural infants (4) Rural children (2) Rural teenagers (3) Rural adults (6)	*E. coli* (24) *S. sonnei* (2)	TpSx
pUK2311	15 (7.9)	Urban infants (4) Urban children (2) Urban teenagers (2) Rural infants (2) Rural children (2) Rural teenagers (1) Rural adults (2)	*E. coli* (13) *S. sonnei* (1)	TpApSxSm
pUK2312	10 (5.3)	Urban teenagers (2) Urban adults (4) Rural infants (3) Rural adults (1)	*E. coli* (10)	TpApSxSm
pUK2317	6 (3.2)	Urban infants (6)	*Klebsiella* spp (6)	TpTcSxSp
pUK2313	5 (2.6)	Urban infants (5)	*E. coli* (5)	TpApSxSm
pUK2307	5 (2.6)	Urban infants (5)	*E. coli* (3) *Klebsiella* spp (2)	TpSx
pUK2302	5 (2.6)	Urban infants (1) Urban children (1) Rural children (2) Rural adults (1)	*E. coli* (5)	TpSx

pUK2314	4 (2.1)	Rural infants (3) Rural adults (1)	*E. coli* (4)	TpApSxSm
pUK2308	4 (2.1)	Rural adults (4)	*Klebsiella* spp (2) *E. coli* (1) *Enterobacter* spp(1)	TpSx
pUK2310	3 (1.6)	Rural children (2) Rural teenagers (1)	*E. coli* (3)	TpSx
pUK2303	2 (1.1)	Urban infants (1) Rural infants (1)	*E. coli* (2)	TpSx
pUK2304	2 (1.1)	Urban infants (2)	*E. coli* (2)	TpSx
pUK2305	2 (1.1)	Rural teenagers (2)	*E. coli* (1) *Klebsiella* spp (1)	TpSx
pUK2306	2 (1.1)	Urban teenagers (1) Rural infants (1)	*E. coli* (2)	TpSx
pUK2309	2 (1.1)	Rural adults (2)	*E. coli* (1) *Klebsiella* spp (1)	TpSx
pUK2315	2 (1.1)	Urban infants (2)	*E. coli* (2)	TpApSxSm
pUK2378	2 (1.1)	Urban infants (1) Urban children (1)	*E. coli* (2)	TpTcApSxSmSp
pUK2370	2 (1.1)	Urban children (1) Rural teenagers (1)	*E. coli* (2)	TpTcApSxSp
pUK2399	2 (1.1)	Rural adults (2)	*E. coli* (1) *Klebsiella* spp (1)	TpTcApGmTnCaz CtxSxSmSpCm

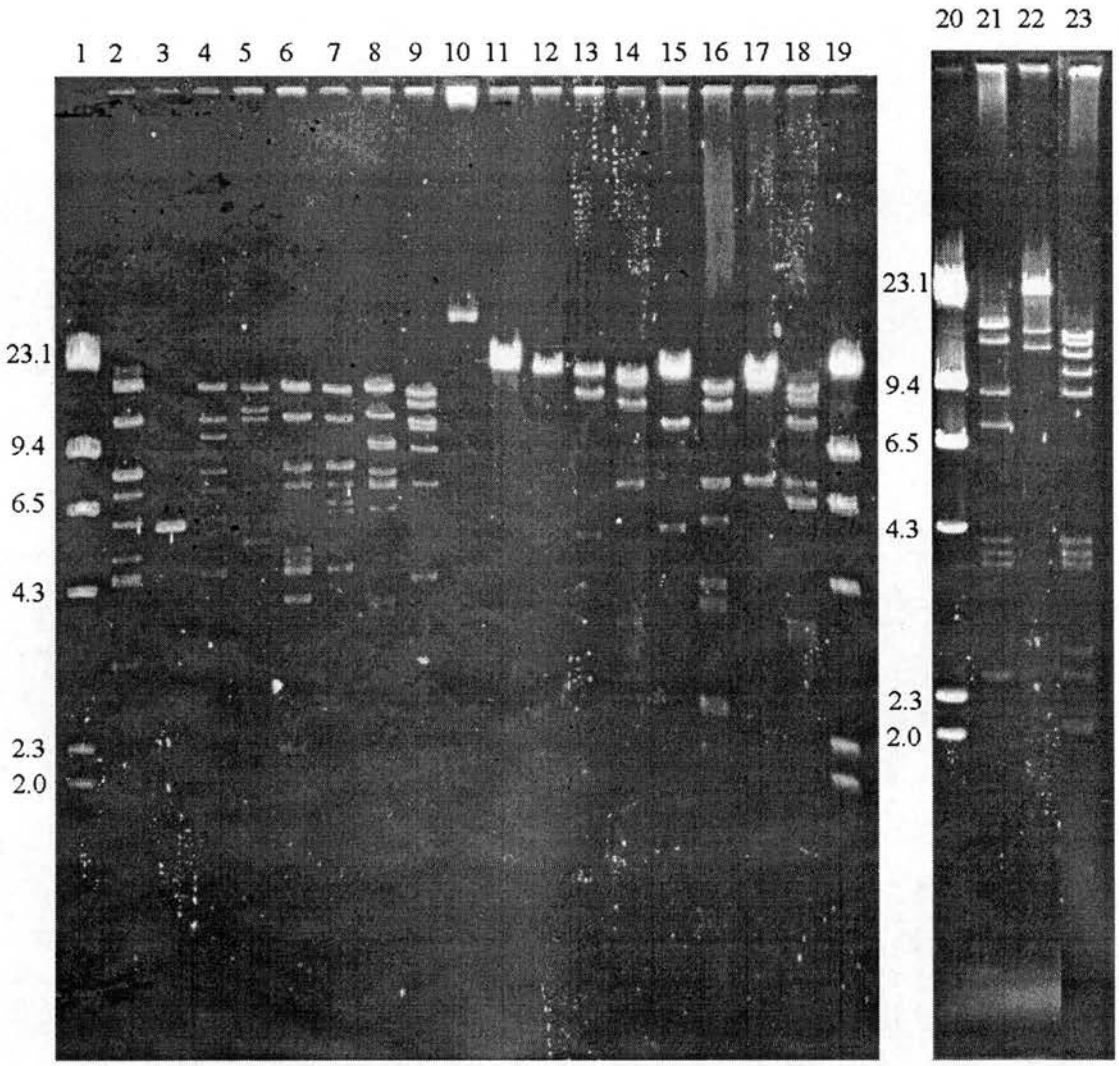


Figure 3.2. A 0.8% agarose gel showing the *Eco*RI restriction profiles of plasmids listed in table 3.3. Lane 1: *Hind*III restricted λ DNA. Lane 2: pUK2301. Lane 3: pUK2302. Lane 4: pUK2303. Lane 5: pUK2304. Lane 6: pUK2305. Lane 7: pUK2306. Lane 8: pUK2307. Lane 9: pUK2310. Lane 10: pUK2308. Lane 11: pUK2309. Lane 12: pUK2308 (Restricted with *Bam*HI). Lane 13: pUK2309 (Restricted with *Bam*HI). Lane 14: pUK2311. Lane 15: pUK2312. Lane 16: pUK2313. Lane 17: pUK2314. Lane 18: pUK2315. Lane 19: *Hind*III restricted λ DNA. Lane 20: *Hind*III restricted λ DNA. Lane 21: pUK2370. Lane 22: pUK2399. Lane 23: pUK2317.

The two most common plasmids were pUK2301 and pUK2311. These two plasmids occurred in 13.8% (26/196), and 7.9% (15/196) of the transconjugants respectively, and were geographically ubiquitous, occurring in all the population groups studied, with the exception of the urban adults from SOWETO. Almost all of these plasmids were isolated from an *E. coli* host with the exception of two isolates of *S. sonnei* which harboured pUK2301 and one isolate of *S. sonnei* which harboured pUK2311. On the other hand, three plasmids pUK2317, pUK2307 and pUK2313 occurred in 3.2% (6/196), 2.6% (5/196) and 2.6% (5/196) of the transconjugants respectively, and were only isolated from one population, urban infants. Plasmids pUK2317 and pUK2313 were harboured only in *Klebsiella* spp. and *E. coli* isolates respectively. Plasmid pUK2307 occurred in more than one species, *E. coli* (n=3) and *Klebsiella* spp. (n=2).

3.7 DNA hybridisation

The oligonucleotide probes discriminated well between the different trimethoprim-resistant DHFRs and no cross-hybridisation was observed with any of the negative control plasmids. The gene probe for the type II DHFRs that was constructed from R388 was shown to hybridise at high stringency (0.05x SSC at 62°C) to the type II DHFRs of R388 and R751 but not R67. At lower stringency, (0.2x SSC at 62°C) this probe hybridised to the control plasmids of most of the resistant DHFR types. Figure 3.3 shows the hybridisation of this probe to the resistant DHFR control plasmids with different stringency washes.

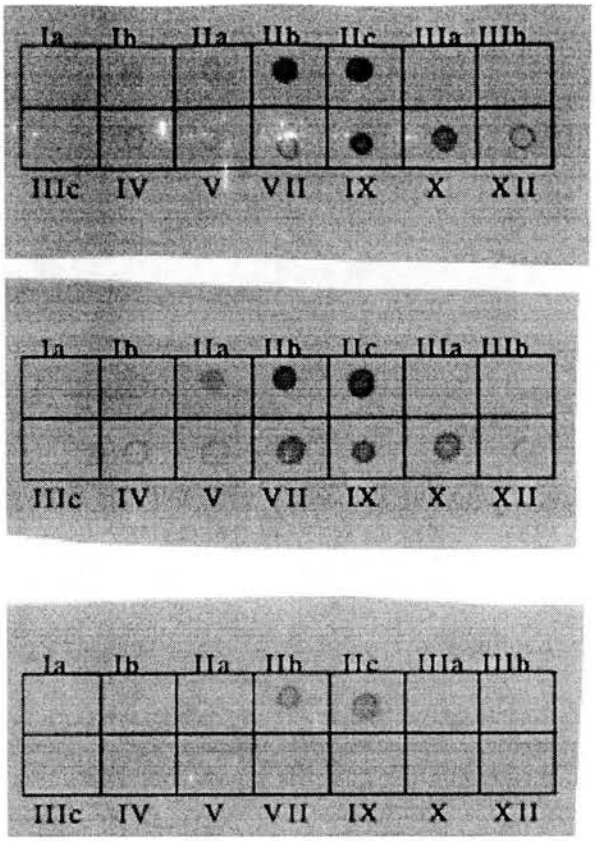


Figure 3.3. Shows the effect of different stringency washes on the hybridisation of the type II gene probe (R388) to the resistant DHFR control plasmids.

3.7.1 Incidence of trimethoprim-resistant DHFR genes in the transconjugants

Plasmid DNA from the 196 isolates that transferred trimethoprim resistance to an *E. coli* J62 recipient was hybridised with the DHFR probes described in 2.10.1 and with the probe for the integrase gene of Tn*21*. The most prevalent DHFR in these plasmids was the type Ib which hybridised to 29.6% (58/196) of the plasmids. Other prevalent DHFRs were the type IIIc/VIII 23% (45/196) and the type V, 12.8% (25/196). The types Ia, VII and XII DHFRs occurred in only 11, 5 and 1 plasmid(s) respectively. None of the isolates hybridised to the intragenic gene probe for DHFRs IIb and IIc that was constructed from R388, or to the oligonucleotide probes for the types IIIa, VI, IX, and X DHFRs. The plasmids of 26.5% (52/196) of the isolates did not hybridise to any of the DHFR probes. The prevalence of each DHFR and the number of different plasmid restriction profiles which hybridised to each DHFR probe

are shown in table 3.7. To determine whether any of the plasmid mediated DHFRs were possibly inserted in a cassette-like manner into an integron structure of a potentially mobile element, the plasmids were probed with an oligonucleotide probe for the integrase gene of Tn*21*. Twenty-one percent (41/196) of the plasmids hybridised to this probe.

Table 3.7. Frequency of DHFR genes and the number of restriction profiles representing each DHFR.

DHFR	No. of plasmids (%)	No. of different restriction profiles†
Type Ia	11 (5.6)	11
Type Ib	58 (29.6)	24 (1)
Type II	0	0
Type IIIa	0	0
Type IIIc/VIII	45 (23)	14
Type V	25 (12.8)	15 (1)
Type VI	0	0
Type VII	5 (2.6)	3 (2)
Type IX	0	0
Type X	0	0
Type XII	1 (0.5)	1
No hybridisation	52 (26.5)	39 (3)
Total	196*	107

*One isolate hybridised with both the type VIII and V DHFR probes.

† The figure in brackets denotes the number of transconjugants in which more than two plasmids occurred and hence no restriction profile could be determined.

Table 3.8 shows the plasmids which hybridised to the probe for the type Ib DHFR gene. The high prevalence of the type Ib was due partially as a result of the presence of the geographically ubiquitous plasmid pUK2301 which occurred in 45% (26/58) of these plasmids. Plasmids pUK2303, pUK2304, pUK2305 and pUK2306 are closely related to and share restriction fragments of similar size to those of pUK2301 (Figure 3.2).

Table 3.8. Plasmids which hybridised to the probe for the type Ib DHFR gene, their resistance profile, and their association with In21.

Plasmid	Isolates	DHFR	In21	Antibiogram
pUK2327	1	Ib	+	Tp
pUK2301	26	Ib	-	TpSx
pUK2302	5	Ib	-	TpSx
pUK2303	2	Ib	-	TpSx
pUK2304	2	Ib	-	TpSx
pUK2305	2	Ib	-	TpSx
pUK2306	2	Ib	-	TpSx
pUK2318	1	Ib	-	TpSx
pUK2319	1	Ib	-	TpSx
pUK2320	1	Ib	-	TpSx
pUK2342	1	Ib	-	TpSx
pUK2321	1	Ib	-	TpSx
pUK2331	1	Ib	-	TpSx
pUK2330	1	Ib	-	TpSx
pUK2333	1	Ib	-	TpSx
pUK2400	1	Ib	+	TpSx
pUK2324	1	Ib	-	TpSx
pUK2354	1	Ib	-	TpAp
pUK2360	1	Ib	+	TpApSx
pUK2366	1	Ib	-	TpApSxSp
pUK2347	1	Ib	+	TpApSxSm
pUK2348	1	Ib	+	TpApSxSm
pUK2351	1	Ib	+	TpApSxSm
pUK2359	1	Ib	+	TpTcApSxSm
not determined*	1	Ib	-	TpSx†

*No restriction pattern was obtained because more than one plasmid was present in the transconjugant. † Antibiogram of the transconjugant.

The majority (49/58) of the plasmids which hybridised to the probe for the type Ib DHFR were resistant to only trimethoprim and sulphonamides and were not associated with the integrase gene of Tn*21*. Five out of seven of the plasmids which were associated with ampicillin resistance hybridised to a probe for the integrase gene of Tn*21*.

The plasmids which hybridised with the probe for the type IIIc/VIII DHFR are shown in table 3.9. Three-quarters of these plasmids were represented by four restriction profiles which accounted for 15/45, 10/45, 5/45 and 4/45 of the plasmids which hybridised with the probe for the type IIIc/VIII DHFR respectively. All the plasmids carried ampicillin resistance determinants, the majority (42/45) of which conferred resistance to sulphonamides and streptomycin. None of these plasmids were carried with the integrase gene of Tn*21*.

Table 3.9. Plasmids which hybridised to the probe for the type VIII DHFR gene, their resistance profile, and their association with In21.

Plasmid	Isolates	DHFR	In21	Antibiogram
pUK2406	1	VIII	-	TpAp
pUK2357	1	VIII	-	TpApSx
pUK2358	1	VIII	-	TpApSx
pUK2311	15	VIII	-	TpApSxSm
pUK2312	10	VIII	-	TpApSxSm
pUK2313	5	VIII	-	TpApSxSm
pUK2314	4	VIII	-	TpApSxSm
pUK2315	2	VIII	-	TpApSxSm
pUK2343	1	VIII	-	TpApSxSm
pUK2344	1	VIII	-	TpApSxSm
pUK2345	1	VIII	-	TpApSxSm
pUK2350	1	VIII	-	TpApSxSm
pUK2352	1	VIII	-	TpApSxSm
pUK2362	1	VIII/V	-	TpApSxSm

The plasmids which hybridised to the probe for the type V DHFR gene are listed in table 3.10. Ubiquitous plasmids carrying the type V DHFR gene were less prevalent in this group of plasmids than those associated with the type Ib and VIII DHFR genes. All the plasmids except one carried resistance to sulphamethoxazole. Resistance determinants associated with these plasmids were: tetracycline, (four plasmids), ampicillin, (four plasmids) and streptomycin (three plasmids). The integrase gene of Tn*21* was associated with 60% (15/25) of these plasmids. None of the plasmids which conferred resistance to ampicillin or streptomycin were associated with the integrase gene of Tn*21*.

Table 3.10. Plasmids which hybridised to the probe for the type V DHFR gene, their resistance profile, and their association with In21.

Plasmid	Isolates	DHFR	In21	Antibiogram
pUK2326	1	V	+	Tp
pUK2307	5	V	-	TpSx
pUK2308	4	V	+	TpSx
pUK2310	3	V	+	TpSx
pUK2309	2	V	+	TpSx
pUK2338	1	V	+	TpSx
pUK2325	1	V	+	TpSx
pUK2323	1	V	+	TpSx
pUK2340	1	V	+	TpTcSx
pUK2339	1	V	+	TpTcSx
pUK2356	1	V	-	TpApSx
pUK2362	1	V/VIII	-	TpApSxSm
pUK2374	1	V	-	TpTcApSxSm
pUK2368	1	V	-	TpTcApCzSxSm
not determined*	1	V	-	TpSx†

*No restriction pattern was obtained because more than one plasmid was present in the transconjugant. † Antibiogram of the transconjugant.

As shown in table 3.11, all eleven plasmids which hybridised to the probe for the type Ia DHFR elaborated a different restriction profile. Only three of these plasmids conferred resistance to sulphonamides one of which was associated with the integrase gene of Tn*21*.

Table 3.11. Plasmids which hybridised to the probe for the type Ia DHFR gene, their resistance profile, and their association with In21.

Plasmid	Isolates	DHFR	In21	Antibiogram
pUK2336	1	Ia	-	Tp
pUK2337	1	Ia	-	TpSmSp
pUK2329	1	Ia	-	TpSmSp
pUK2334	1	Ia	-	TpSmSp
pUK2408	1	Ia	-	TpSmSp
pUK2353	1	Ia	-	TpAp
pUK2405	1	Ia	-	TpAp
pUK2355	1	Ia	-	TpAp
pUK2349	1	Ia	-	TpApSxSm
pUK2346	1	Ia	-	TpApSxSp
pUK2364	1	Ia	+	TpApSxSmSp

Of the five transconjugants which hybridised to the type VII DHFR probe, two harboured more than one plasmid and, as a result no restriction profiles could be obtained for these isolates. The remaining three transconjugants had unique restriction profiles. The resistance markers harboured by these transconjugants are shown in table 3.12. All of these plasmids hybridised to the probe for the integrase gene of Tn*21*.

Only one transconjugant harbouring plasmid pUK2316 hybridised to the type XII DHFR probe. This plasmid conferred resistance to trimethoprim, streptomycin and spectinomycin, and hybridised with the probe for the integrase gene of Tn*21*.

Table 3.12. Plasmids which hybridised to the probe for the type VII DHFR gene, their resistance profile, and their association with In21.

Plasmid	Isolates	DHFR	In21	Antibiogram
pUK2405	1	VII	+	TpApSx
pUK2382	1	VII	+	TpApTcSxSm
pUK2402	1	VII	+	TpApTcSxSmCm
not determined*	2	VII	+	TpApTcSxSm†

*No restriction patterns was obtained because more than one plasmid was present in the transconjugants. † Antibiogram of the transconjugants.

The association of the resistant DHFRs with the minimum inhibitory concentrations of trimethoprim expressed by the transconjugants is shown in table 3.13. The transconjugants which hybridised to probes for the DHFRs type Ia, Ib, V, VII and XII were all resistant to high concentrations of trimethoprim (MIC $\geq$ 1024mg/l). Of the transconjugants which hybridised to the type VIII DHFR probe, 62% (28/45) conferred resistance to intermediate concentrations of trimethoprim (MIC<1024mg/l). All the transconjugants which did not hybridise to any of the DHFR probes were resistant to high concentrations of trimethoprim (MIC $\geq$ 1024mg/l), the majority of which (87%) were resistant to >2048mg/l of trimethoprim.

Table 3.13. Trimethoprim MIC for each DHFR type.

	Trimethoprim MIC (mg/l)					
DHFR	>2048	2048	1024	516	256	128
Type Ia	9	1	1	-	-	-
Type Ib	18	29	11	-	-	-
Type VIII	1	7	9	17	10	1
Type V	15	9	1	-	-	-
Type VII	2	1	2	-	-	-
Type XII	1	-	-	-	-	-
No hybridisation	45	6	1	-	-	-

3.7.2 Prevalence of non-transferable DHFRs

Of the 357 isolates of trimethoprim-resistant Gram-negative commensal faecal flora, resistance was transferable to a recipient strain *E. coli* J62 in a liquid mating in 196 (55%) of the isolates. Total DNA from the 161 (45.1%) isolates, which did not transfer their resistance to a recipient strain, was probed for the presence of the types Ia, Ib, IIb, IIc, IIIa, V, VI, VII, VIII, IX, X and XII DHFR genes. The frequency of hybridisation of the DHFR gene probes to the isolates which did not transfer trimethoprim resistance by conjugation to an *E. coli* J62 recipient strain, is shown in table 3.14.

Table 3.14. Frequency of resistant DHFRs in isolates which did not transfer trimethoprim resistance to an *E. coli* J62 recipient.

DHFR	No. of isolates (%)	% of total isolates
Type Ia	41 (25.5)	11.5
Type Ib	20 (12.4)	5.6
Type IIb/IIc	0	0
Type IIIa	0	0
Type IIIc/VIII	2 (1.2)	0.6
Type V	3 (1.9)	0.8
Type VI	0	0
Type VII	62 (38.5)	17.4
Type IX	0	0
Type X	0	0
Type XII	0	0
No hybridisation	38 (23.6)	10.6
Total	161	45%

Of the isolates which did not transfer their resistance, the most prevalent DHFR type was the type VII which occurred in 38.5% (62/161) of the isolates. The second most prevalent DHFR was the type Ia 25.4% (41/161), followed by the type Ib 12.4% (20/161), the type V, three isolates and the VIII, two isolates. The species distribution of the isolates, which hybridised to each probe is shown in table 3.15. The type Ia DHFR probe hybridised to DNA from the widest range of different species. These included *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter freundii* and *Proteus vulgaris*. Five isolates hybridised to more than one DHFR probe, four of which

hybridised to both the type Ia and VII DHFR probes and one isolate to the types Ia and VIII DHFR probes. Since there was no cross hybridisation with the control strains, one possibility is that two resistant DHFR genes are present in these isolates. None of the isolates hybridised to the probes for the types II, IIIa, VI, IX and XII DHFR genes. Thirty-eight isolates did not hybridise to any of the DHFR probes.

Table 3.15. Frequency of non-transferable DHFRs and the organism of origin.

DHFR	Hybridisation (%)	Species
Type Ia	41* (25.5)	*E. coli* (32)
		Klebsiella sp (3)
		Enterobacter sp (4)
		Citrobacter freundii (1)
		Proteus vulgaris (1)
Type Ib	20 (12.4)	*E. coli* (14)
		Klebsiella sp (6)
Type V	3 (1.9)	*E. coli* (1)
		Klebsiella sp (2)
Type VII	62 (38.5)	*E. coli* (57)
		Klebsiella sp (3)
		Enterobacter sp (2)
Type VIII	1 (0.6)	*Citrobacter freundii* (1)
No hybridisation	38 (23.6)	*E. coli* (30)
		Klebsiella sp (6)
		Enterobacter sp (1)
		Pseudomonas aeruginosa (1)

*Of the isolates which hybridised to the type Ia probe, four also hybridised with the type VII probe and one hybridised with the type VIII probe.

3.7.3 Genetic location of non-transferable resistant DHFRs

To determine the genetic location of the resistant DHFR genes, representative isolates carrying each DHFR type were randomly selected. Plasmid and total DNA from each isolate was separated on a 0.8% agarose gel, Southern blotted and probed to determine the genetic location of the DHFR gene. The type VII DHFR gene was found located on the chromosome in all the isolates tested (n=9). Figure 3.4 shows the agarose gel and the Southern blot of seven of these isolates. Sixteen isolates harbouring a non-transferable type VII DHFR gene including these nine isolates were further analysed by Southern blotting a *Bam*HI restriction of the chromosomal DNA and probing it with oligonucleotide probes for the type VII DHFR (Figure 3.5) and the integrase gene of Tn*21* (results not shown). For each isolate both probes hybridised to fragments of the same size. This suggests that in these isolates, the DHFR gene was located within an integron which is integrated into the chromosome of the host bacterium.

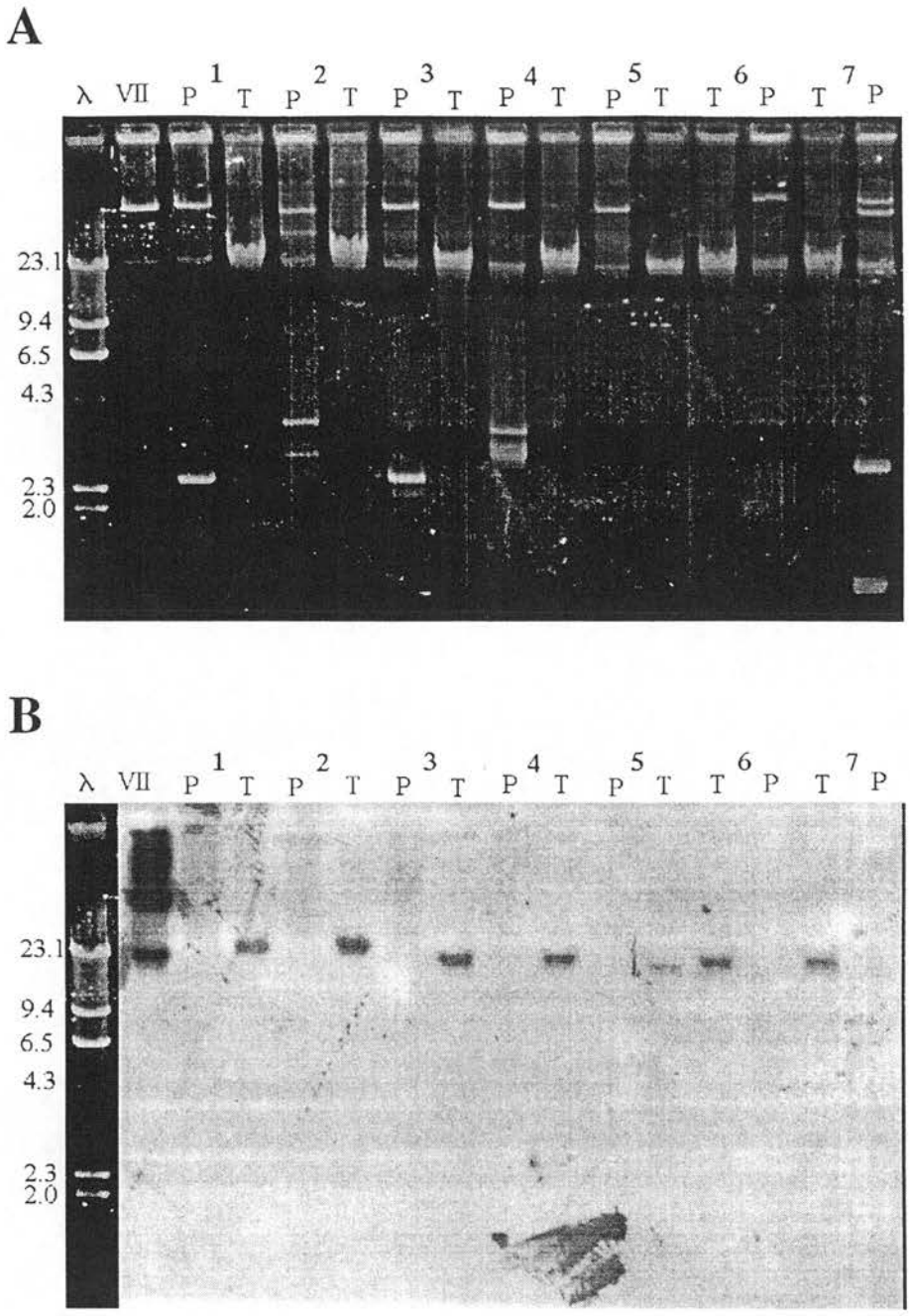


Figure 3.4. (A) 0.8% Agarose gel of plasmid and total DNA preparations from seven isolates which hybridised to the probe for the type VII DHFR. (B) Southern blot showing the chromosomal location of the type VII DHFR gene. λ-*Hind* III restricted λ DNA; VII-Plasmid pLMO226 control; P-Plasmid DNA; T-Total DNA.

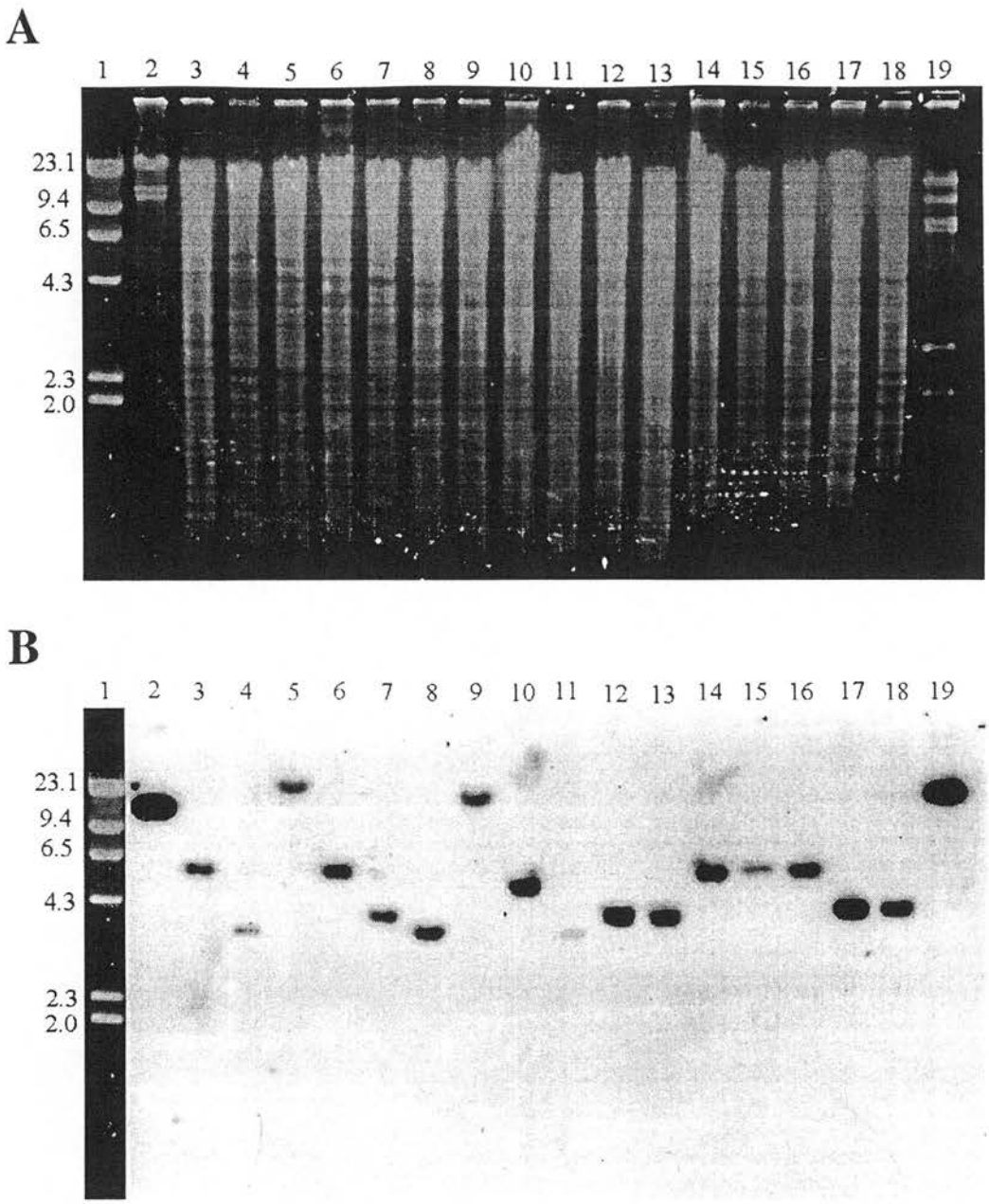


Figure 3.5. (A) 1% Agarose gel of restricted total DNA preparations from isolates which hybridised to the probe for the type VII DHFR. (B) Southern blot showing the fragment sizes which hybridised to the type VII DHFR probe. Lane 1, *Hind* III restricted λ DNA; Lane 2, *Bam*H I restricted pLMO226 control DNA; Lanes 3-18, *Bam*H I restricted total DNA from isolates which hybridised to the type VII DHFR probe; 19, *Eco*R I restricted pLMO226 control DNA.

Plasmid and chromosomal DNA from nine isolates which hybridised to the type Ia DHFR probe, but could not transfer resistance, were selected for Southern hybridisation. The chromosomal DNA probed positive in eight out of nine isolates which hybridised to the type Ia DHFR probe (Figure 3.6). In the remaining isolate the type Ia DHFR was located on a plasmid. Since the electrophoretic mobility of this plasmid is similar to that of the chromosomal DNA, it cannot be accurately determined whether the DHFR might also be located on the chromosome. A Southern blot of *Eco*RI restricted chromosomal DNA from these isolates revealed a number of fragments of variable size which hybridised to the type Ia DHFR probe (Figure 3.7). The probe for the integrase gene of Tn*7* hybridised to fragments of the same size in all of the chromosomal mediated *dhfrIa* bearing isolates (results not shown). The plasmid carrying the type Ia DHFR gene probed positive for the integrase gene of Tn*21* (Table 3.16). Attempts to mobilise this plasmid into a restriction endonuclease deficient recipient (*E. coli* K802) in a filter mating at 22°C and 37°C were not successful.

Southern hybridisations of plasmid and total DNA from seven isolates that probed positive for the type Ib DHFR gene revealed that the type Ib DHFR gene in these isolates was plasmid mediated (Figure 3.8). A small 9kb plasmid was present in five out of seven of these isolates. The two larger plasmids hybridised to the integrase gene of Tn*21* (results not shown). All three isolates which hybridised to the type V DHFR probe were found to be plasmid-mediated (Figure 3.9) and hybridised with the integrase gene of Tn*21* (results not shown). None of these plasmids bearing the type Ib and V DHFR genes could be mobilised into *E. coli* K802 in a filter mating at 22°C and 37°C.

Table 3.16. Genetic location of DHFR genes and associated integrase genes.

DHFR	(n)	Location of DHFR	Associated transposon
Type Ia	9	Chromosome (8)	Tn*7* (8)
		Plasmid (1)	Tn*21*(1)
Type Ib	7	Plasmid (7)	Tn*21*(2)
Type V	3	Plasmid (3)	Tn*21* (3)
Type VII	9	Chromosome (9)	Tn*21* (9)

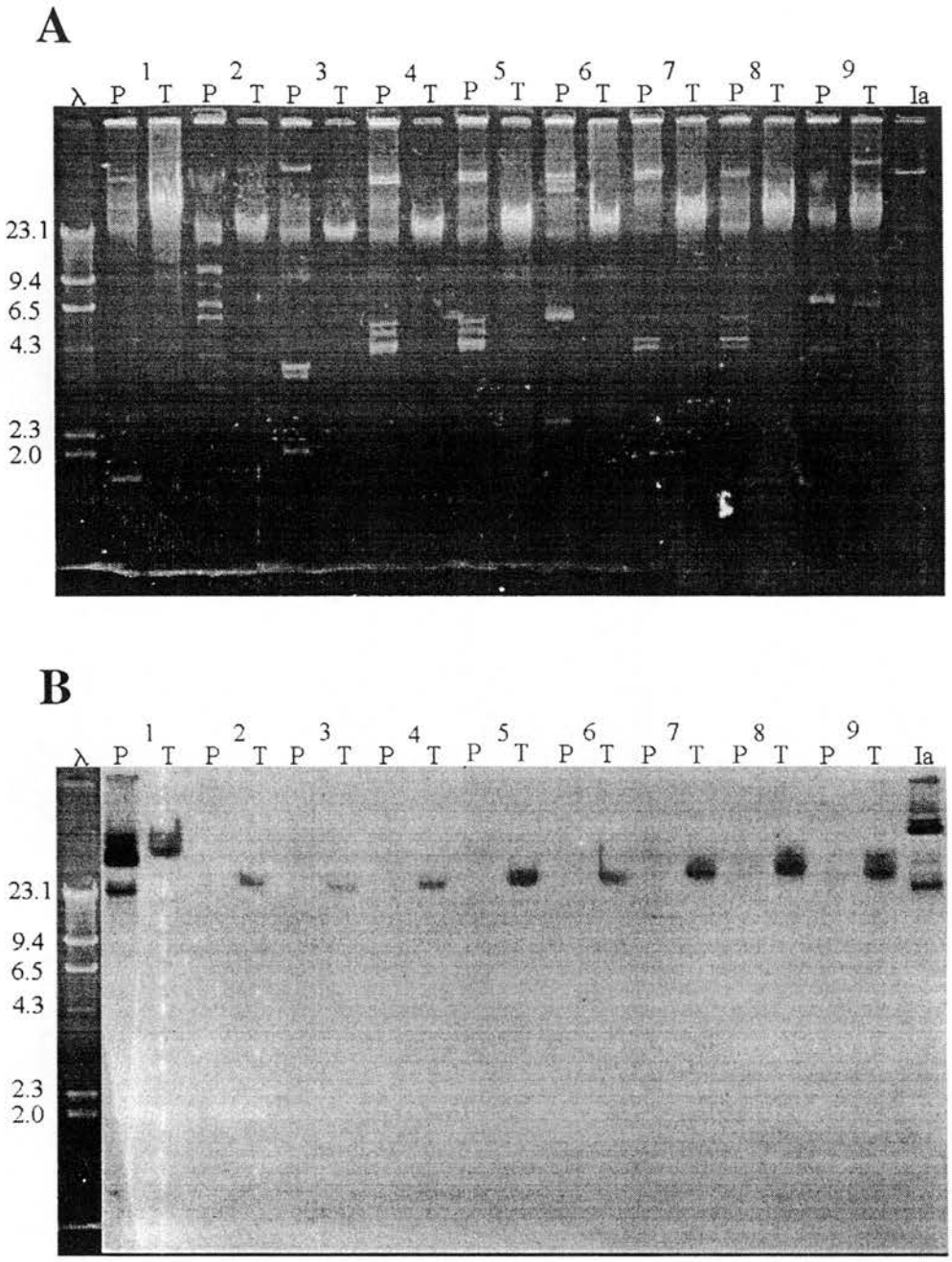


Figure 3.6. (A) 0.8% Agarose gel of plasmid and total DNA preparations from nine isolates which hybridised to the probe for the type Ia DHFR. (B) Southern blot showing the plasmid and chromosomal location of the type Ia DHFR gene. P-Plasmid DNA; T-Total DNA; Ia-Plasmid pFE506 control.

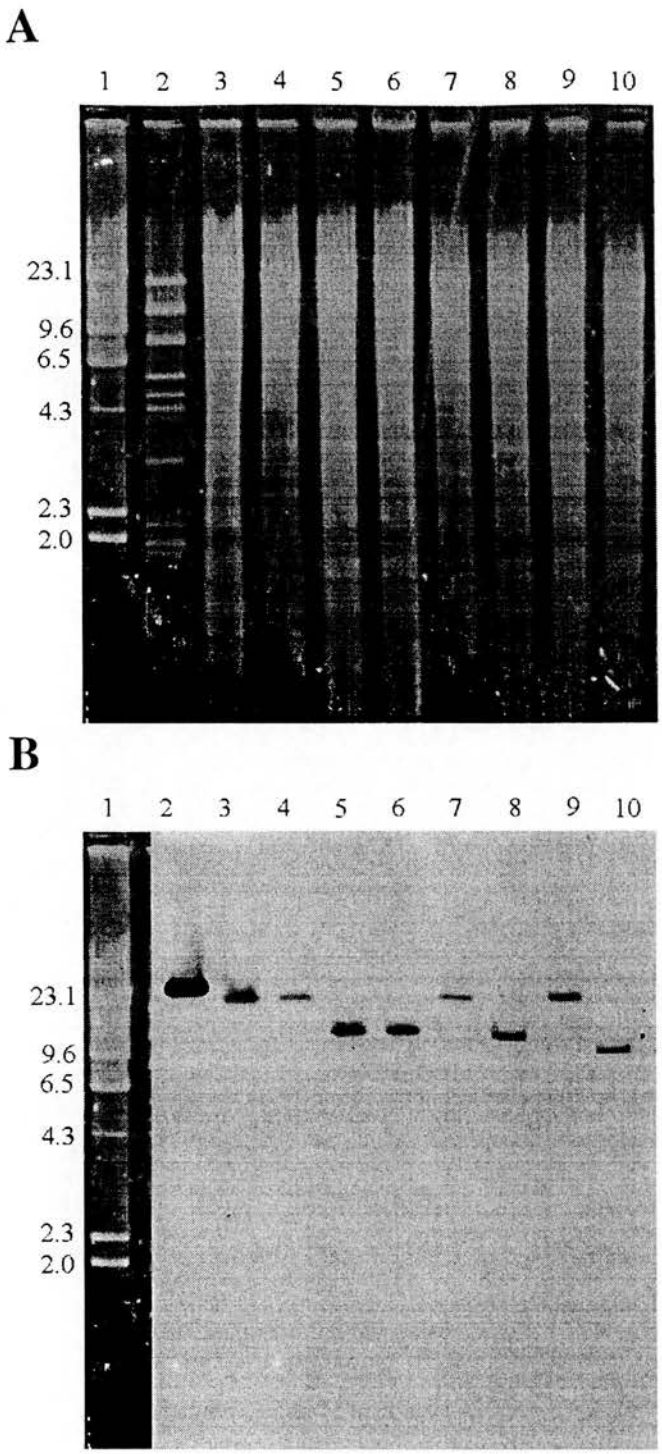


Figure 3.7. (A) 1% Agarose gel of restricted total DNA preparations from eight isolates which hybridised to the probe for the type Ia DHFR. (B) Southern blot showing the fragment sizes which hybridised to the type Ia DHFR probe. Lane 1: *Hind* III restricted λ DNA. Lane 2: *Eco*R I restricted pFE506 control DNA. Lanes 3-10: *Eco*R I restricted total DNA from eight isolates which hybridised to the type Ia DHFR probe.

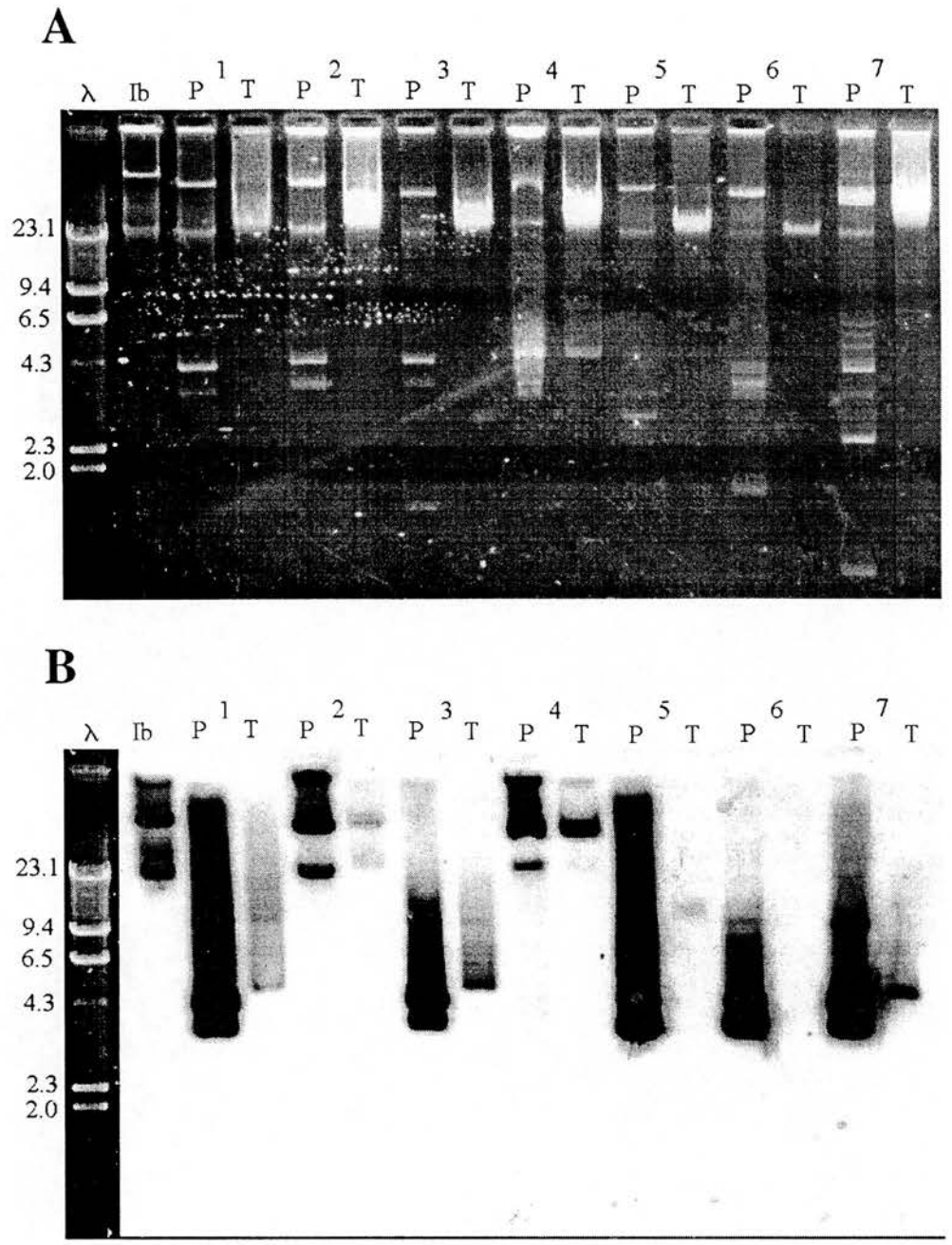


Figure 3.8. (A) 0.8% Agarose gel of plasmid and total DNA preparations from seven isolates which hybridised to the probe for the type Ib DHFR. (B) Southern blot showing the plasmid location of the type Ib DHFR gene. λ-*Hind* III restricted λ DNA; Ib-Plasmid pUK163 control; P-Plasmid DNA; T-Total DNA.

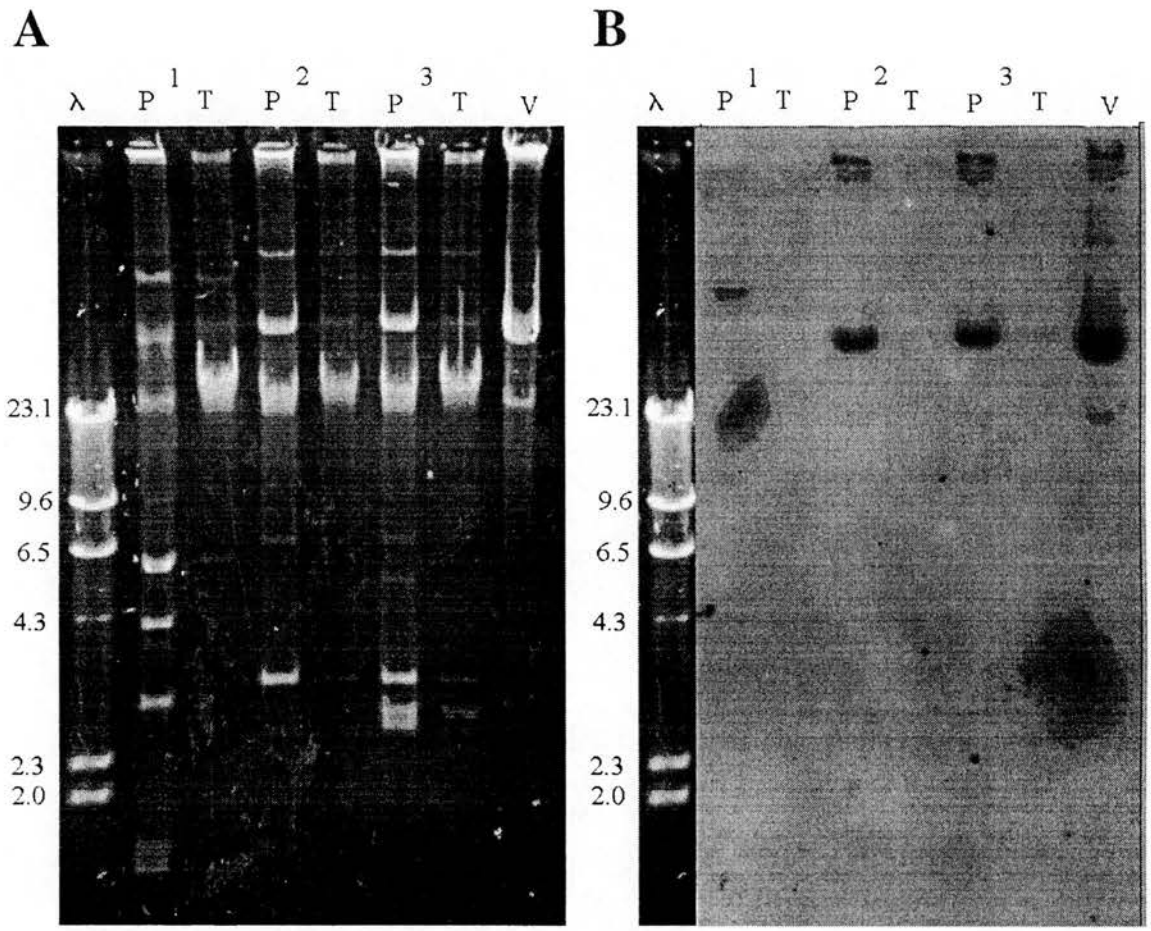


Figure 3.9. (A) 0.8% Agarose gel of plasmid and total DNA preparations from three isolates which hybridised to the probe for the type V DHFR. (B) Southern blot showing the plasmid location of the type V DHFR gene. P-Plasmid DNA; T-Total DNA; V-Plasmid pLMO20 control.

3.8 Detection of a novel trimethoprim resistance determinant

The plasmids which did not hybridise to any of the probes for the resistant DHFR genes were sorted according to their restriction profiles and resistance markers (Table 3.17). Only three plasmids occurred in two or more transconjugants. Of these plasmids, the most frequently isolated was pUK2317 which occurred in six isolates of *Klebsiella* spp., all isolated from a crèche in SOWETO. Plasmid pUK2378 was isolated from two different urban populations, and both isolates carrying pUK2399 were isolated from rural adults.

Despite the lack of geographically ubiquitous plasmids harbouring the unknown resistant DHFR genes, many of these plasmids shared identical antimicrobial resistance profiles. The restriction profiles of some of these plasmids elaborated several restriction fragments of similar molecular weight indicating that some homology existed between some of the plasmids. Two groups emerged from these comparisons. The largest group comprised twelve plasmids which conferred resistance to trimethoprim, tetracycline, ampicillin, sulphonamides, streptomycin, spectinomycin and chloramphenicol but did not hybridise to the probe for the integrase gene of Tn*21*. The restriction profiles of eight of these plasmids (pUK2384, pUK2385, pUK2387, pUK2388, pUK2392, pUK2394, pUK2395 and pUK2396) shared some homology (Figure 3.10). Two plasmids, pUK2386 and pUK2389 of this resistance group also shared a few restriction fragments similar molecular weight (Figure 3.10).

The second group of six plasmids hybridised to the probe for the integrase gene of Tn*21* and conferred resistance to trimethoprim, tetracycline, ampicillin, sulphonamides, streptomycin and spectinomycin. The plasmids pUK2378, pUK2380 and pUK2381 from this group shared several restriction fragments of similar molecular weight and are shown in figure 3.10. Plasmid pUK2385 from the first group and plasmid pUK2381 from the second group were selected for cloning and further study.

Table 3.17. Plasmids conferring unknown trimethoprim-resistant DHFRs, their resistance profile, and their association with In21.

Plasmid	Isolates	In21	Antibiogram
pUK2326	1	-	Tp
pUK2322	1	-	TpSx
pUK2342	1	-	TpSx
pUK2363	1	-	TpSxSp
pUK2341	1	-	TpSmSp
pUK2328	1	+	TpTcSp
pUK2317	6	-	TpTcSxSp
pUK2335	1	-	TpTcGmSx
pUK2369	1	-	TpTcApSxSp
pUK2370	1	-	TpTcApSxSp
pUK2403	1	-	TpTcApStSp
pUK2367	1	+	TpApSxSmSp
pUK2365	1	-	TpApSxSmSp
pUK2371	1	+	TpTcApSxSm
pUK2372	1	-	TpTcApSxSm
pUK2373	1	+	TpTcApSxSm
pUK2361	1	+	TpApSxSmSpCm
pUK2376	1	-	TpTcApSxSmSp
pUK2375	1	+	TpTcApSxSmSp
pUK2378	2	+	TpTcApSxSmSp
pUK2379	1	+	TpTcApSxSmSp
pUK2380	1	+	TpTcApSxSmSp
pUK2381	1	+	TpTcApSxSmSp
pUK2383	1	-	TpTcApSxStCm
pUK2384	1	-	TpTcApSxSmSpCm
pUK2385	1	-	TpTcApSxSmSpCm
pUK2404	1	-	TpTcApSxSmSpCm
pUK2387	1	-	TpTcApSxSmSpCm
pUK2388	1	-	TpTcApSxSmSpCm
pUK2395	1	-	TpTcApSxSmSpCm
pUK2396	1	-	TpTcApSxSmSpCm
pUK2391	1	+	TpTcApSxSmSpCm
pUK2392	1	-	TpTcApSxSmSpCm
pUK2394	1	-	TpTcApSxSmSpCm
pUK2386	1	-	TpTcApSxSmSpCm
pUK2389	1	-	TpTcApSxSmSpCm
pUK2390	1	-	TpTcApSxSmSpCm
pUK2397	1	-	TpTcApGmSxSmStSpCm
pUK2398	1	-	TpTcApGmTnCazSxSmStSpCm
pUK2399	2	-	TpTcApGmTnCazCtxSxSmStSpCm
not determined	1	+	TpTcApSxCm
not determined	1	-	TpApSxSmCm
not determined	1	-	TpTcApSxSmSpCm

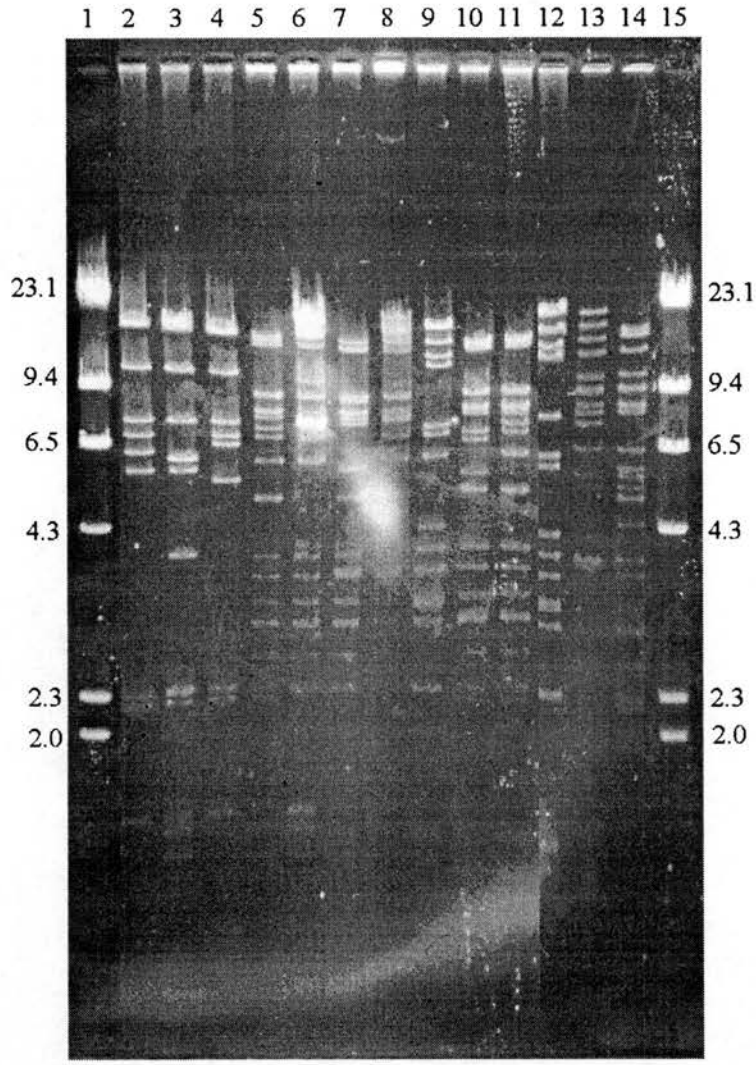


Figure 3.10. A 0.8% agarose gel showing the *Eco*RI restriction profiles of plasmids which harbour unknown DHFR genes. Lane 1: *Hind*III restricted λ DNA. Lane 2: pUK2378. Lane 3: pUK2380. Lane 4: pUK2381. Lane 5: pUK2384. Lane 6: pUK2385. Lane 7: pUK2387. Lane 8: pUK2388. Lane 9: pUK2392. Lane 10: pUK2394. Lane 11: pUK2395. Lane 12: pUK2396. Lane 13: pUK2386. Lane 14: pUK2389. Lane 15: *Hind*III restricted λ DNA.

3.8.1 Cloning the DHFR

Purified DNA of pUK2385 and pUK2381 was restricted with *Pst*I, *Bam*HI, and *Eco*RI and ligated into appropriately restricted pUC18. No resistant clones were derived from pUK2385 after complete digestion with any of these restriction enzymes. The trimethoprim-resistant DHFR of the 70kb plasmid pUK2381 as determined by the cumulative sizes of the restricted fragments, could be cloned into pUC18 either as a 3.3kb *Bam*HI fragment, or as a 4.5kb *Pst*I fragment. The restriction map of the 3.3kb *Bam*HI fragment of pUK2381 which expressed a resistant DHFR gene that was cloned into pUC18 (pUK2408) is shown in figure 3.11. Plasmid pUK2408 was restricted with *Eco*RI, and the large fragment was religated to produce pUK2409. The subsequent cloning strategy used to produce the 1225-bp *Pvu*II/*Hinc*II fragment from pUK2409 that was sub-cloned into the *Sma*I site of pUC18 to produce plasmid pUK2410 is shown in figure 3.11.

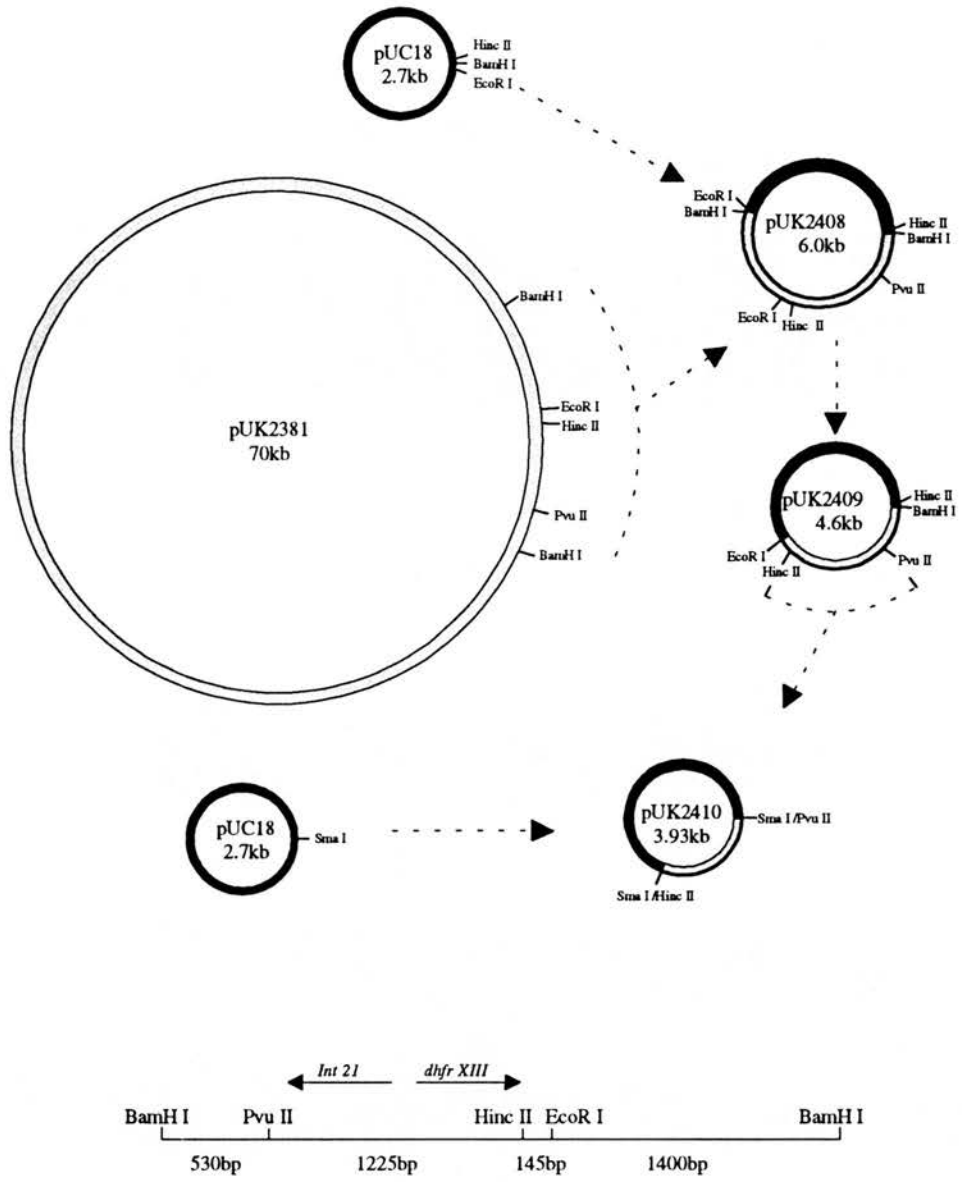


Figure 3.11. The restriction map of the 3.3kb *Bam*HI fragment of pUK2381 and the strategy of cloning plasmids pUK2408, pUK2409 and pUK2410.

3.8.2 Nucleotide Sequence

The nucleotide sequence of the novel DHFR was determined from plasmid pUK2410. Figure 3.12 shows the direction of sequencing of the cloned fragment by primer walking. Table 3.18 shows the sequences of the oligonucleotide primers internal to the M13 primers.

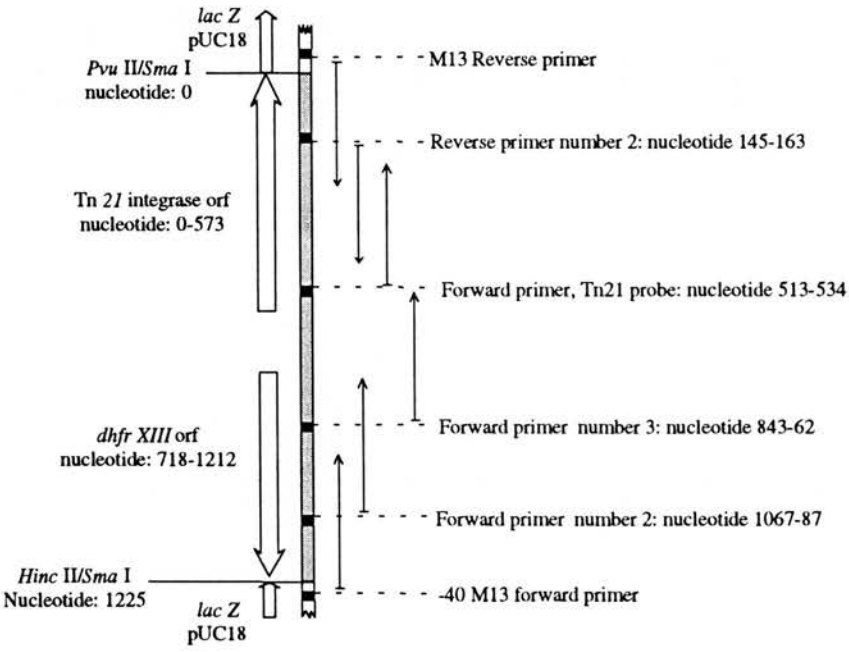


Figure 3.12 The direction of sequencing of the 1225-bp *Pvu*II/*Hinc*II fragment of pUK2381.

Table 3.18. Oligonucleotide primers used in sequencing reactions.

Primer	Nucleotides	Oligonucleotide sequence
Reverse # 2	145-163	5'-CGTTCCATACAGAAGCTGG-3'
Forward # 4	513-534	5'-GTCAAGGTTCTGGACCAGTTGC-3'
Forward # 3	843-862	5'-CCATAACGACCACTTTGCTC-3'
Forward # 2	1067-1087	5'-GTACCTCAGATAGAAAGACG-3'

The nucleotide sequence of the cloned section of pUK2410 was compared with all the bacterial DNA sequences in the GenBank and EMBL databases. The region from nucleotide 0 to 573 was identical to that of the first 191 amino acids of the open reading frame of the integrase gene of Tn*21* and other Tn*21*-like-transposons such as those carrying the resistance genes *dhfrV* (Sundström *et al* 1988), *dhfrXII* (Heikkilä *et al* 1993), OXA-2 borne on R46 (Hall and Vockler 1987), *dhfrVII* borne on Tn*5086* (Sundström *et al* 1993) and In0 from pVS1 (Bissonnette and Roy 1992).

Downstream of the In21 ORF was a 498-bp ORF potentially encoding a polypeptide of 165 amino acids. The start of the ORF began with an ATG start codon at positions 718-720 (Figure 3.13). The ORF terminated with a TAA stop codon (positions 1213 to 1215, figure 3.13). The ORF showed a high degree of homology (85%) the nucleotide sequence of the type XII DHFR (Singh *et al* 1992b; Heikkilä *et al* 1993.

The upward arrow in figure 3.13 marks the sequence (GTTPuPu) a few bases upstream of the DHFR ATG start codon, and indicates the point of site-specific-insertion of the DHFR gene cassette at the end of the integron specific sequence. The 135-bp region from nucleotide 573-708 that occurs between the integrase ORF and the novel DHFR gene cassette was almost identical to the integron specific sequences of Tn*21*-like elements located upstream of resistance genes located in a cassette-like manner within an integron. These identical sequences contain the site for integration of resistance gene cassettes, the promoter for these cassettes, and the start of the integrase gene.

Figure 3.13. (Following Page) Nucleotide sequence of *dhfrXIII* and the upstream flanking region of the *int* gene of Tn*21*. The vertical arrow indicates the GTTPuPu sequence which occurs at the beginning of the *dhfrXIII* cassette. Asterisk denotes stop codon.

Pvu II

```
CTGCTCGCGC AGGCTGGGTG CCAAGCTCTC GGGTAACATC AAGGCCCGAT     50
CCTTGGAGCC CTTGCCCTCC CGCACGATGA TCGTGCCGTG ATCGAAATCC    100
AGATCCTTGA CCCGCAGTTG CAAACCCTCA CTGATCCGCA TGCCCGTTCC    150
ATACAGAAGC TGGGCGAACA AACGATGCTC GCCTTCCAGA AAACCGAGGA    200
TGCGAACCAC TTCATCCGGG GTCAGCACCA CCGGCAAGCG CCGCGACGGC    250
CGAGGTCTTC CGATCTCCTG AAGCCAGGGC AGATCCGTGC ACAGCACCTT    300
GCCGTAGAAG AACAGCAAGG CCGCCAATGC CTGACGATGC GTGGAGACCG    350
AAACCTTGCG CTCGTTCGCC AGCCAGGACA GAAATGCCTC GACTTCGCTG    400
CTGCCCAAGG TTGCCGGGTG ACGCACACCG TGGAAACGGA TGAAGGCACG    450
AACCCAGTGG ACATAAGCCT GTTCGGTTCG TAAGCTGTAA TGCAAGTAGC    500
GTATGCGCTC ACGCAACTGG TCCAGAACCT TGACCGAACG CAGCGGTGGT    550

                        ←int start
AACGGCGCAG TGGCGGTTTT CATGGCTTGT TATGACTGTT TTTTTGTACA    600
GTCTATGCCT CGGGCATCCA AGCAGCAAGC GCGTTACGCC GTGGGTCGAT    650
GTTTGATGTT ATGGAGCAGC AACGATGTTA CGCAGCAGGG CAGTCGCCCT    700
AAAACAAAGT TAGCCGT  ATG AAC CCG GAA TCG GTC CGC ATT TAT    744
        ↑           Met Asn Pro Glu Ser Val Arg Ile Tyr     9

CTG GTC GCT GCC ATG GGT GCC AAT CGG GTT ATT GGC AAT GGT    786
Leu Val Ala Ala Met Gly Ala Asn Arg Val Ile Gly Asn Gly    23

CCC GAT ATC CCC TGG AAA ATC CCA GGT GAG CAG AAG ATT TTT    828
Pro Asp Ile Pro Trp Lys Ile Pro Gly Glu Gln Lys Ile Phe    37

CGC AGG CTC ACC GAG AGC AAA GTG GTC GTT ATG GGC CGC AAG    870
Arg Arg Leu Thr Glu Ser Lys Val Val Val Met Gly Arg Lys    51

ACA TTT GAG TCC ATA GGC AAG CCC TTA CCA AAC CGC CAC ACA    912
Thr Phe Glu Ser Ile Gly Lys Pro Leu Pro Asn Arg His Thr    65

GTG GTG CTC TCG CGC CAA GCT GGT TAT AGC GCT CCT GGT TGT    954
Val Val Leu Ser Arg Gln Ala Gly Tyr Ser Ala Pro Gly Cys    79

GCA GTT GTT TCA ACG CTG TCA CAC GTA TCG CCA TCG ACA GCC    996
Ala Val Val Ser Thr Leu Ser His Val Ser Pro Ser Thr Ala    93

GAA CAC GGC AAA GAA CTC TAC GTA GCG CGC GGA GCC GAG GTA   1038
Glu His Gly Lys Glu Leu Tyr Val Ala Arg Gly Ala Glu Val   107

TAT GCG CTG GCG CTA CCG CAT GCC AAC GGC GTC TTT CTA TCT   1080
Tyr Ala Leu Ala Leu Pro His Ala Asn Gly Val Phe Leu Ser   121

GAG GTA CAT CAA ACC TTT GAG GGT GAC GCC TTC TTC CCA GTG   1122
Glu Val His Gln Thr Phe Glu Gly Asp Ala Phe Phe Pro Val   134

CTT AAC GCA GCA GAA TTC GAG GTT GTC TCA TCC GAA ACC ATT   1164
Leu Asn Ala Ala Glu Phe Glu Val Val Ser Ser Glu Thr Ile   149

CAA GGC ACA ATC ACG TAC ACG CAC TCC GTC TAT GCG CGT CGT   1206
Gln Gly Thr Ile Thr Tyr Thr His Ser Val Tyr Ala Arg Arg   163

AAC GGC TAA CAAGTCCGTC                                   1225
Asn Gly  *          Hinc II                              165
```

The translated polypeptide for the DHFR is shown in figure 3.13. The novel DHFR gene has been tentatively named *dhfrXIII* and the encoded polypeptide, the type XIII DHFR (EBML accession number Z50802 ECIN1DHFR). The amino acid sequence was compared with the sequence of all the other amino acid sequences in the SwissProt database. The 31 best scores were all DHFR genes. The amino acid sequence of the type XIII DHFR shared the highest identity with the type XII DHFR (82.4%). With the exception of the type XII DHFR, homology between the type XIII DHFR and the other DHFRs ranged between 15 and 36%.

3.9 Biochemical properties of the type XIII DHFR

3.9.1 Specific DHFR activity

The specific DHFR activity of crude protein extracts from, the wild-type *E. coli* isolate RA33-2 which harboured plasmid pUK2381 that expresses *dhfrXIII*, the corresponding J62-2 transconjugant; the pUC18 clone of *dhfrXIII* (pUK2410) and the plasmid host strains *E. coli* J62-2 and HB101 are shown in table 3.19.

Table 3.19. Specific activity of the strains harbouring the type XII DHFR.

Host strain	Plasmid	Specific activity *
E. coli J62-2	-	1.40
E. coli HB101	-	0.88
E. coli RA33-2	pUK2381	4.04
E. coli J62-2	pUK2381	2.79
E. coli HB101	pUK2410	30.07

* Specific activity expressed as nmol DHF reduced /min/mg protein.

As a result of the expression of pUK2381 encoded *dhfrXIII*, the increase in the specific DHFR activity produced by the resistant DHFR in the transconjugant was equivalent to that produced by the J62-2 host chromosomal DHFR. As a result of the high copy number of pUC18 the pUK2410 clone of *dhfrXIII* produced an approximately thirty-four-fold increase in the specific DHFR activity in comparison to the *E. coli* HB101 host chromosomal DHFR.

3.9.2 Sephadex gel-exclusion chromatography

The crude protein extract of HB101 harbouring pUK2410 was precipitated by ammonium sulphate saturation. The 50-80% saturated fraction contained most of the DHFR activity, and was virtually free of NADPH oxidase activity. This fraction was dialysed and applied to a Sephadex G75 column. Figure 3.14 shows the OD_{280} of the fractions of the molecular weight standards and the DHFR activity of the eluted sample measured by the change in OD_{340}.

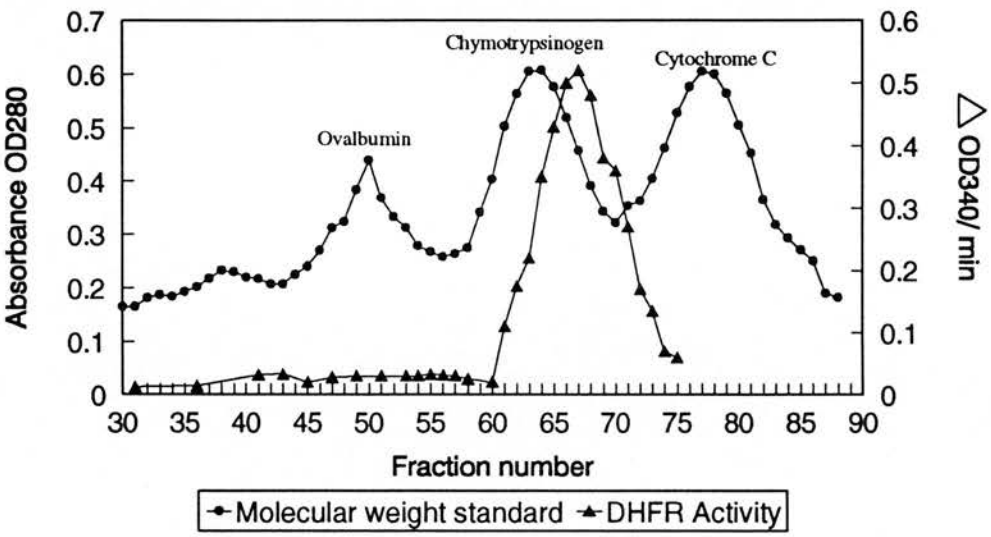


Figure 3.14. The OD_{280} of the fractions of the molecular weight standards and the change in OD_{340} of the eluted sample.

The peak fractions of the molecular weight standards and the peak DHFR activity was plotted on a graph (Figure 3.15) and from the graph, the molecular weight of the type XIII DHFR was estimated to be 20.9 KDa.

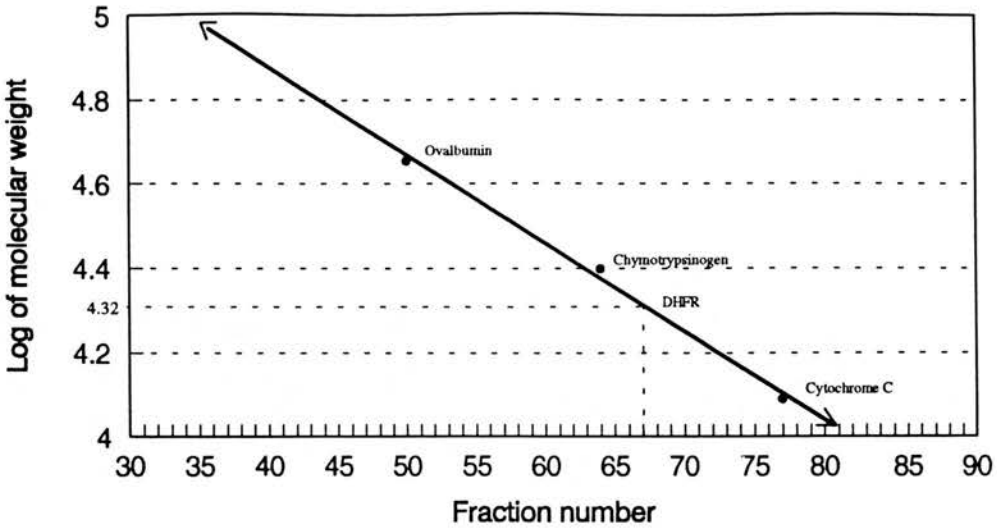


Figure 3.15. Estimation of molecular weight of the type XIII DHFR.

3.9.3 Drug inhibition

The activity of the type XIII DHFR was assayed in the presence of increasing concentrations of trimethoprim (Figure 3.16) and methotrexate (Figure 3.17).

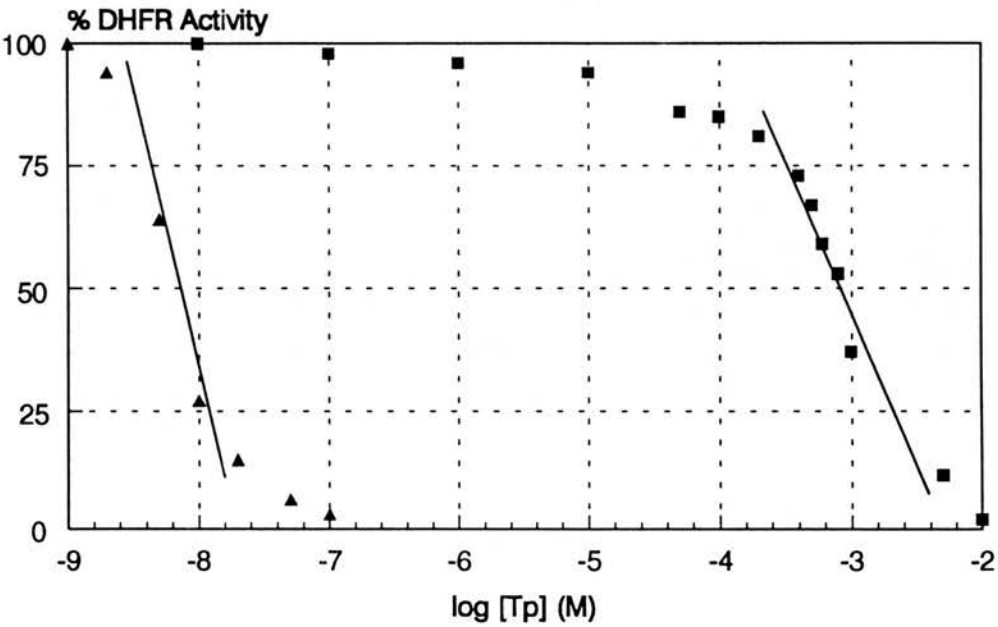


Figure 3.16. The percentage activity of partially purified type XIII DHFR (■) and the chromosomal DHFR from *E. coli* (HB101) (Δ) in the presence of increasing concentrations of trimethoprim.

The concentration of trimethoprim required to inhibit the activity of the type XIII DHFR by 50% (ID_{50}) was extremely high (800μM) and in comparison to the chromosomal DHFR of *E. coli* (HB101) (ID_{50}=0.007μM) the type XIII DHFR was more than 100 000x more resistant to trimethoprim. The type XIII DHFR was 1 700x more resistant to inhibition by methotrexate (ID_{50}=5μM) than the chromosomal DHFR of *E. coli* (HB101) (ID_{50}=0.003μM). However, the ID_{50} for methotrexate was low enough to suggest that the binding site of dihydrofolate was not dissimilar to that of other DHFR genes.

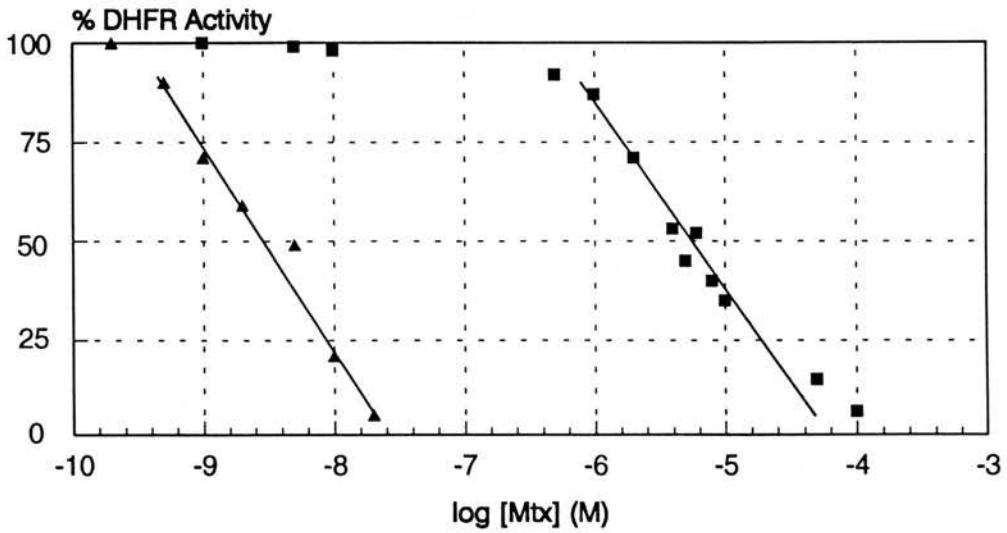


Figure 3.17. The percentage activity of partially purified type XIII DHFR (■) and the chromosomal DHFR from *E. coli* (HB101) (Δ) in the presence of increasing concentrations of methotrexate.

3.9.4 Temperature sensitivity

The loss of DHFR activity of the type XIII DHFR as a result of exposure to 45°C is shown in figure 3.18. The time taken to inhibit DHFR activity by 50% (TD_{50}) was approximately seven minutes.

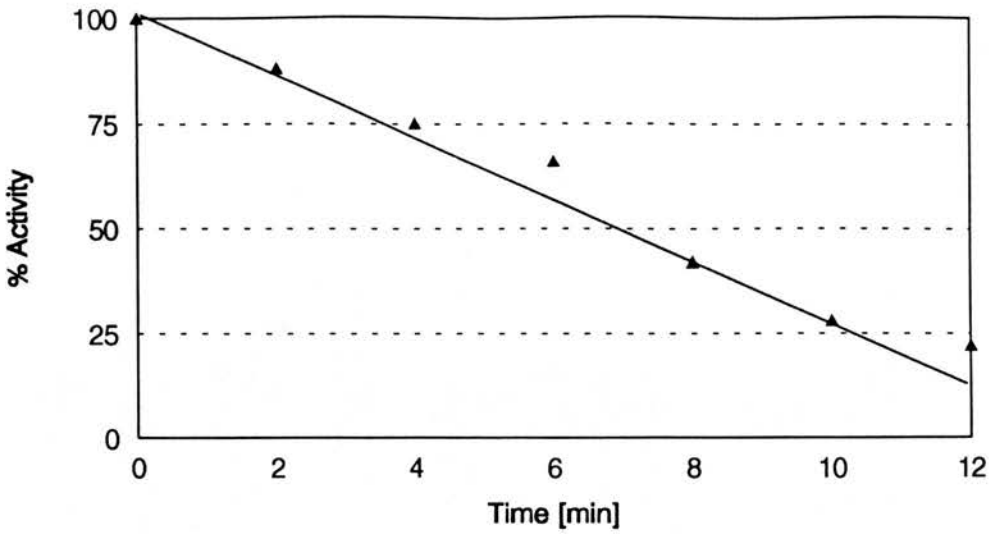


Figure 3.18. Temperature inhibition of the type XIII DHFR at 45°C.

3.9.5 Michaelis and trimethoprim inhibition constant

The Lineweaver-Burke plots used to determine the Michaelis constant and the inhibitor constant for trimethoprim are shown in figure 3.19.

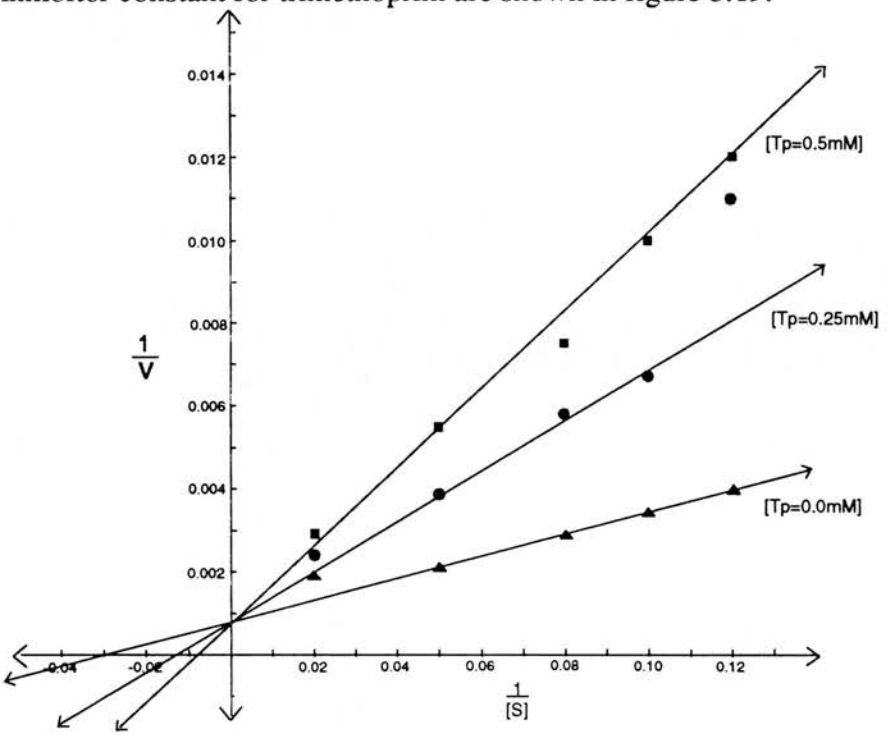


Figure 3.19. Lineweaver-Burke plots: the reciprocal of the dihydrofolate concentration (1/[S]) is plotted against the reciprocal of the rate of DHFR activity (1/V) in the presence and absence of trimethoprim. (Δ) No trimethoprim. (●)250μM trimethoprim. (■) 500μM trimethoprim.

From the intercepts on the X-axis of the plots (the most wayward point of each series was ignored), the K_m for the type XIII DHFR was calculated as (1/-0.03)=33.3μM DHF. The apparent K_m values in the presence of trimethoprim (K_p) were (1/-0.0125)=80μM and (1/-0.008)=125μM at trimethoprim concentrations of 250μM and 500μM respectively. From the equation in 2.12.4 the K_i values at these trimethoprim concentrations were 178μM and 182μM respectively (Mean K_i =180μM DHF).

3.10 Prevalence of the type XIII DHFR

3.10.1 Development of a *dhfrXIII* specific probe

The nucleotide sequences of the closely related DHFRs XII and XIII were aligned to determine regions of maximum heterogeneity (Figure 3.20). The least homologous region between these two genes (10-bp over a 23-bp stretch) occurred between nucleotides 218 to 240. This region overlapped the region from which the oligonucleotide probe for *dhfrXII* was selected (underlined in figure 3.20). A 30mer oligonucleotide probe 5'-AAGCTGGTTATAGCGCTCCTGGTTGTGCAG-3' from the identical region from which the *dhfrXII* probe was derived was used as the probe for *dhfrXIII*.

3.10.2 DNA hybridisation

Of the 90 isolates which did not hybridise to the probes for any of the DHFR genes, 50% (45/90) hybridised to the probe for the type XIII DHFR. Fifty-eight percent (30/52) of self transmissible plasmids, hybridised to the probe for *dhfrXIII*. Of the isolates which did not transfer trimethoprim resistance 39% (15/38) hybridised to the probe for the type XIII DHFR. The plasmids which hybridised to the probe for the type XIII DHFR are shown in table 3.20.

Multiple resistance was extremely prevalent amongst the plasmids which harboured the type XIII DHFR. Most of these plasmids conferred resistance to five or more antimicrobial agents. Resistance to ampicillin, tetracycline, sulphonamides and streptomycin was virtually ubiquitous. Spectinomycin resistance occurred in (23/30) of the plasmids, and resistance to chloramphenicol occurred in (17/30) of the plasmids. Forty percent (12/30) of the isolates hybridised to a probe for the integrase

gene of Tn*21*. With the exception of one isolate with an MIC of 2048 mg/l, the MICs of trimethoprim conferred by these plasmids were greater than 2048 mg/l.

```
                                                                      50
dhfrxiii ATGAACCCGGAATCGGTCCGCATTTATCTGGTCGCTGCCATGGGTGCCAA
         |||||| ||||||| || |||||||||| || ||||| ||||| |||||
dhfrxii  ATGAACTCGGAATCAGTACGCATTTATCTCGTTGCTGCGATGGGAGCCAA
                                                                     100
dhfrxiii TCGGGTTATTGGCAATGGTCCCGATATCCCCTGGAAAATCCCAGGTGAGC
         ||||||||||||||||||||  ||||||||||||||| || |||||||
dhfrxii  TCGGGTTATTGGCAATGGTCCTAATATCCCCTGGAAAATTCCGGGTGAGC
                                                                     150
dhfrxiii AGAAGATTTTTCGCAGGCTCACCGAGAGCAAAGTGGTCGTTATGGGCCGC
         |||||||||||||||| ||||| ||| | ||||| || || ||||| ||
dhfrxii  AGAAGATTTTTCGCAGACTCACTGAGGGAAAAGTCGTTGTCATGGGGCGA
                                                                     200
dhfrxiii AAGACATTTGAGTCCATAGGCAAGCCCTTACCAAACCGCCACACAGTGGT
         ||||| |||||||| || |||||||| |||| ||||| |||||| ||||
dhfrxii  AAGACCTTTGAGTCTATCGGCAAGCCTCTACCGAACCGTCACACATTGGT
                                                                     250
dhfrxiii GCTCTCGCGCCAAGCTGGTTATAGCGCTCCTGGTTGTGCAGTTGTTTCAA
          || || |||||||||   ||  ||||  |||| || | ||||||||||
dhfrxii  AATCTCACGCCAAGCTAACTACCGCGCCACTGGCTGCGTAGTTGTTTCAA
                                                                     300
dhfrxiii CGCTGTCACACG-TATCGCCATCGACAGCCGAACACGGCAAAGAACTCTAC
         ||||||| |||| ||||| | | | || |||||| |||||| |||||||||
dhfrxii  CGCTGTCGCACGCTATCG-CTTTGGCATCCGAACTCGGCAATGAACTCTAC
                                                                     350
dhfrxiii GTAGCGCGCGGAGCCGAGGTATATGCGCTGGCGCTACCGCATGCCAACGG
         || ||| ||||||| ||| |||| | ||||| ||||| || ||| ||||
dhfrxii  GTCGCGGGCGGAGCTGAGATATACACTCTGGCACTACCTCACGCCCACGG
                                                                     400
dhfrxiii CGTCTTTCTATCTGAGGTACATCAAACCTTTGAGGGTGACGCCTTCTTCC
         ||| ||||||||||||||||||||||| ||||||||||||||||||
dhfrxii  CGTGTTTCTATCTGAGGTACATCAAACCTTCGAGGGTGACGCCTTCTTCC
                                                                     450
dhfrxiii CAGTGCTTAACGCAGCAGAATTCGAGGTTGTCTCATCCGAAACCATTCAA
         || |||| |||| | |||||||||| |||||||| ||||||||||||||
dhfrxii  CAATGCTCAACGAAACAGAATTCGAGCTTGTCTCAACCGAAACCATTCAA
                                                                    498
dhfrxiii GGCACAATCACGTACACGCACTCCGTCTATGCGCGTCGTAACGGCTAA
         |    |||  ||||||| |||||||| |||||||||| |||||||||
dhfrxii  GCTGTAATTTCCGTACACCCACTCCGTTTATGCGCGCGAAACGGCTAA
```

Figure 3.20. The alignment of the nucleotide sequence of the types XII and XIII DHFR genes: the underlined region shows the heterogeneous region from which the oligonucleotide probes for the respective DHFR genes were selected.

Table 3.20. Plasmids which hybridised to the probe for the type XIII DHFR gene, their resistance profile, their association with In21 and the identity of the plasmid donor .

Plasmid	Plasmid donor	Isolates	In21	Antibiogram
pUK2328	*Enterobacter* spp.	1	+	TpTcSp
pUK2367	*E. coli*	1	+	TpApSxSmSp
pUK2365	*Kluyvera* spp.	1	-	TpApSxSmSp
pUK2361	*Klebsiella* spp.	1	+	TpApSxSmSpCm
pUK2371	*E. coli*	1	+	TpTcApSxSm
pUK2372	*E. coli*	1	-	TpTcApSxSm
pUK2373	*E. coli*	1	+	TpTcApSxSm
pUK2376	*E. coli*	1	-	TpTcApSxSmSp
pUK2375	*E. coli*	1	+	TpTcApSxSmSp
pUK2378	*E. coli*	2	+	TpTcApSxSmSp
pUK2379	*E. coli*	1	+	TpTcApSxSmSp
pUK2380	*E. coli*	1	+	TpTcApSxSmSp
pUK2381	*E. coli*	1	+	TpTcApSxSmSp
pUK2384	*E. coli*	1	-	TpTcApSxSmCm
pUK2385	*E. coli*	1	-	TpTcApSxSmCm
pUK2404	*Klebsiella* spp.	1	-	TpTcApSxSmSpCm
pUK2387	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2388	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2395	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2396	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2391	*Klebsiella* spp.	1	+	TpTcApSxSmSpCm
pUK2392	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2394	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2386	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2389	*E. coli*	1	-	TpTcApSxSmSpCm
pUK2390	*E. coli*	1	-	TpTcApSxSmSpCm
not determined*	*E. coli*	1	-	TpApSxSmCm†
not determined*	*E. coli*	1	+	TpTcApSxCm†
not determined*	*E. coli*	1	-	TpTcApSxSmSpCm†

*No restriction patterns was obtained because more than one plasmid was present in the transconjugant. † Antibiogram of the transconjugant.

Of the isolates which hybridised to the type XIII DHFR probe, but did not transfer trimethoprim resistance, the majority were *E. coli* (11/15). The remaining isolates were *Klebsiella* spp. (3/15) and *Enterobacter* spp. (1/15). All these isolates were resistant to more than 2048 mg/l of trimethoprim with exception of one *E. coli* (MIC=2048mg/l) and one *Enterobacter* spp. (MIC=256 mg/l).

4.0 DISCUSSION

"In the beginners mind there are many possibilities. In the experts mind there are few."

Shunryu Suzuki - Zen Mind, Beginners Mind

The high incidence of trimethoprim resistance in Gram-negative commensal faecal flora in South Africa (74%) is similar to that experienced in other developing countries such as China (61%), Venezuela (64%) (Lester *et al* 1990), Bangladesh (45%) (Mamun *et al* 1993) and India (98%) (Amyes *et al* 1992a). This has challenged a number of theories which relate to our understanding of the reasons for the high levels of resistance in commensal faecal organisms in developing countries. For instance, South Africa is fairly unique amongst developing countries in that there is no 'over the counter' policy for the sale and distribution of antimicrobial agents and, as a result, the abuse of these agents that is associated with this phenomena is restricted. This suggests that 'over the counter' policies play a minor role in encouraging the development of resistance in a population. Since the water supply in South African populations studied was not contaminated with resistant organisms, it appears that this is not a major contributing factor towards the spread of resistant organisms as suggested by Young (1993). The single common factor shared between the developing countries studied, is a low standard of living which is usually associated with overcrowding, poor sanitation and a low standard of personal hygiene. This is supported by work by Reves *et al* (1990), who has shown that overcrowding, and the low levels of hygiene associated with infants attending day care centres were major factors contributing to the spread of resistant organisms. Ignorance concerning the use of antimicrobials is a factor not restricted to developing countries.

The Gram-negative aerobic bacilli commonly found in stool specimens of healthy individuals are *Escherichia coli, Klebsiella, Enterobacter, Citrobacter, Proteus and Pseudomonas* species (Jawetz *et al* 1982). In this study, trimethoprim resistance was recorded in all the above species as well as *Klyvera* spp. and *Shigella sonnei* isolates. The high incidence of resistance associated with these organisms is of great concern since these organisms are often implicated as the source of infection in urinary tract, enteric and other opportunistic infections (Brumfitt *et al* 1971; Bettelheim *et al* 1971). The relationship between the incidence of resistance in commensal flora and clinical

isolates is reflected in a number of studies. In developed countries, the incidence of trimethoprim resistance in hospital isolates ranges from 5-20% (Brumfitt *et al* 1983; Maskell 1983; Mayer *et al* 1985; Kraft *et al* 1985; Huovinen and Toivanen 1986; Towner and Slack 1986; Heikkilä *et al* 1990a; Harnett 1992a) and likewise, the corresponding figure for commensal flora is similar 3-25% (Bonten *et al* 1990; Levy *et al* 1988; Shanahan *et al* 1994). In developing countries a similar relationship exists between the incidence of resistance in commensal flora and clinical isolates; in South India 64% of Gram-negative bacteria isolated from urinary infections were resistant to trimethoprim (Young *et al* 1986b) and, in the same study area, faecal carriage of resistant commensal organisms was ubiquitous (Amyes *et al* 1992a). In South Africa the incidence of trimethoprim resistance is lower than in South India; however a similar relationship exists, with 38%, 53% and 60% resistance being recorded in clinical isolates from three different centres (Wylie and Koornhof 1989) compared with 74% resistance in commensal faecal isolates.

The incidence of resistance associated with trimethoprim resistance in commensal and clinical isolates and their plasmids from various studies is shown in table 4.1. In South African faecal isolates and plasmids, chloramphenicol and gentamicin resistance associated with trimethoprim resistance occurred less frequently than in any of the other surveys of clinical and commensal isolates from both developed and developing countries. The incidence of resistance to ampicillin, tetracycline, streptomycin and chloramphenicol, associated with trimethoprim in plasmids isolated from faecal isolates, was lower in South Africa than in South India. In contrast, sulphonamide and spectinomycin resistance co-transferred on plasmids more frequently in the South African isolates. The relationship between the associated resistance factors of faecal and urinary isolates in the Indian isolates was similar, with a slightly higher incidence of resistance in the clinical isolates. This pattern differed in Nigeria, where associated resistance was more prevalent in plasmids from diarrhoeal isolates than urinary isolates. In South Africa, this relationship cannot be determined since there are no published data available for the incidence of resistance associated with trimethoprim resistance in clinical isolates from South Africa. Furthermore comparisons are difficult to make since the break-points/ disc concentrations and media employed in the testing differed between the studies.

A further concern regarding the high prevalence of trimethoprim and associated resistance factors in commensal flora is the potential for the horizontal spread of these resistance factors between members of the *Enterobacteriaceae* including potential

Table 4.1. Resistance associated with trimethoprim resistance in isolates of commensal and pathogenic organisms and their plasmids.

		% Resistance							
Country, year	Isolates	Sx	Tc	Ap	Sm	Sp	Gm	Cm	Reference
Developed countries									
Ontario, Canada 1990	*E. coli* UTI	100	83	87	-	-	-	43	Harnett (1992a)
London, UK 1978-79	Clinical plasmids	48	28	60	59	-	-	32	Towner *et al* (1980)
Paris, France 1981-84	UTI plasmids	94	-	75-90	75-90	-	-	65-80	Papadopoulou *et al* (1986)
Athens, Greece 1989	UTI Plasmids	44	57	47	23	20	20	39	Tsakris *et al* (1991)
Developing countries									
Mexico, 1981	Faecal *E. coli*	88	90	80	94	-	-	52	Murray *et al* (1982)
Chile, 1983	*E. coli* UTI	100	53	88	100	-	6	69	Murray *et al* (1985)
Chile, 1983	*E. coli* UTI, Plasmids	100	0	71	100	-	0	29	Murray *et al* (1985)
Chile, 1983-84	Enterobacteria Plasmids	71	82	72	47	-	71	82	Urbina *et al* (1989)
Bangkok,Thailand,1984	*E. coli* UTI	95	93	85	97	-	18	77	Murray *et al* (1985)
Bangkok,Thailand,1984	*E. coli* UTI, Plasmids	75	50	50	75	-	0	50	Murray *et al* (1985)
Nigeria, 1987	Hospital UTIs	100	72	86	69	46	21	62	Lamikanra and Ndep (1989)
Nigeria, 1987	Hospital UTI, Plasmids	63	18	47	35	16	13	39	Lamikanra and Ndep (1989)
Nigeria, 1988	*E. coli* diarrhoea Plasmids	85	91	60	66	78	82	70	Lamikanra *et al* (1990)
South India, 1984	UTI	99	96	98	100	45	55	85	Young *et al* (1986b)
South India, 1984	UTI, Plasmids	84	43	76	71	41	43	47	Young *et al* (1986b)
South India, 1990	Faecal flora	95	92	77	97	38	-	60	Tait and Amyes (1994)
South India, 1990	Faecal, Plasmids	58	67	70	72	22	-	19	Tait and Amyes (1994)
South Africa, 1992	Faecal flora	-	88	72	-	-	3.1	-	This Study
South Africa, 1992	Faecal, Plasmids	91	30	56	48	24	2.6	12	This Study

pathogens such as *Salmonella* spp., *Shigella* spp. and enteropathogenic *E. coli*. In the isolates studied, the frequency of transfer of trimethoprim resistance by conjugation (55%) occurred within the normal range of 35-75% demonstrated in several studies of clinical and commensal isolates of *Enterobacteriaceae* (Chugh 1985; Young *et al* 1986b; Urbina *et al* 1989; Lamikanra and Ndep 1989; Lamikanra *et al* 1990; Tsakris *et al* 1991; Harnett 1992a). Of the 107 different plasmid restriction profiles that were demonstrated in the transconjugants, seven plasmids occurred in more than one species of Enterobacteria suggesting that horizontal transfer of plasmids conferring trimethoprim resistance occurs between different commensal species. Furthermore, two of these plasmids occurred in both *E. coli* and potentially pathogenic *S. sonnei* isolates. Although there is no evidence to determine in which species these plasmids originally evolved, the high prevalence of these two plasmids in the commensal flora (26/357 and 15/357) suggests that these plasmids may have evolved and dispersed in the commensal flora and then spread to the *S. sonnei* isolates. The presence of these plasmids reinforce the importance of the commensal flora as a reservoir of resistance genes.

The advantage of studying commensal faecal flora is that one can obtain a broader sample of the gene pool of resistant organisms, since the collection of clinical isolates is often associated with a number of biases. For example, sporadic outbreaks of infection caused by one particular organism as described by Barg *et al* (1990) and Voogd *et al* (1992) can result in an underestimation of the size of the pool of resistance genes. Isolates from in-patients are also associated with sample biases, since these usually contain a number of repeat specimens (Huovinen 1986) and, furthermore, a large proportion of the infections of inpatients are acquired from the resident flora within the hospital itself (Wingard *et al* 1993). Huovinen (1984) found that the incidence of trimethoprim resistance in urinary tract infections, in inpatients at a Finnish hospital, was double that in catheter related infections. This suggests that the patients were susceptible to colonisation by the more resistant resident hospital flora.

The localised spread of resistant strains and/ or plasmids was only evident in two of the populations. Three plasmids, which were harboured by more than one isolate, were isolated only from the rural adults. Notably, the rural adults were all associated with the hospital at Shongwe Mission and, consequently, were in contact with patients. This particular population consisted of healthy mothers feeding sick children, mothers attending a nutrition centre for children and finally escorts to patients visiting

the out-patients department. One of these plasmids pUK2399 conferred resistance to most classes of antimicrobials including third-generation cephalosporins and was resistant to TpTcApGmTnCazCtxSxSmSpCm. Since aminoglycosides and third-generation cephalosporins are usually restricted to hospital use it is likely that this plasmid originated from within the hospital. Five other plasmids which were harboured by more than one isolate were isolated only from the urban infants. This group was made up of individuals attending either a childminder or a crèche in SOWETO. The most prevalent of these plasmids occurred in six *Klebsiella* spp. isolates which exhibited identical biochemical and antimicrobial resistance profiles, suggesting that this was the same isolate which has spread within the childminder or crèche. A similar phenomenon has been observed in day care centres in Texas by Reves *et al* (1987) and Singh *et al* (1992b).

Gene probing for trimethoprim-resistant DHFRs has been plagued with a number of problems which can potentially result in erroneous data. The most significant setback being the nature of the DHFR gene probes. The high degree of homology found in the conserved regions of DHFRs especially in the type I like enzymes (Ia, Ib, V, VI, VII) and the type XII and XIII DHFRs suggest that it is essential to use accurate oligonucleotide probes for the detection and discrimination between different DHFR genes. The use of DHFR gene probes containing regions which extend upstream or downstream of the DHFR can lead to false positive identifications of DHFRs. This is because many different DHFRs are found as cassette-like structures in the integration site of Tn*21*. There may, therefore, be hybridisation of the gene probe with the flanking regions of the integron; for example the region of Tn*21* which surrounds the type IIb DHFR on R388 is almost identical to that of the type V DHFR of pLMO20 (Sundström *et al* 1988). The following DHFRs have been observed to contain the characteristic repeats that suggest that the gene forms part of a cassette: Ia, IIa, IIb, IIc, V, VI, VII (Sundström *et al* 1993), the type Ib (Young *et al* 1994), the type XII (Heikkilä *et al* 1993) and the type XIII (this thesis). As a result of the use of trimethoprim in the form of co-trimoxazole, DHFR genes are often associated with the *sulI* gene which predominantly occurs as a gene cassette within an integron (Rådström *et al* 1991). The close association between these two genes can result in sections of the *sulI* gene being incorporated into a DHFR gene probe. This may increase the incidence of false positives in the gene probing of DHFRs and thus underestimate the variability of the DHFRs in a population. The use of the highly specific oligonucleotide probes in this study, in conjunction with control strains for all

the published plasmid and transposon mediated DHFRs are far less likely to produce compromised data on the prevalence and distribution of DHFR genes.

The distribution of DHFR genes in South Africa is distinctly different from that recorded in any previous study. The type Ia which has been shown to be the most prevalent DHFR in Europe (Figure 1.7), occurred in only 14.6% of the South African isolates. This is not unusual, since a low prevalence of this DHFR gene has been recorded in most studies conducted outside Europe including Sri Lanka (Sundström *et al* 1987), USA (Singh *et al* 1992b), India (Tait and Amyes 1994) and Thailand (Chatkaeomorakot *et al* 1987).

Despite the low prevalence of the type Ia DHFR, the molecular epidemiology of this DHFR is very similar to that recorded at other centres. As shown in table 4.2, trimethoprim resistance was not transferable by conjugation in the majority of the isolates which harboured the type Ia DHFR. In most of the isolates tested, the type Ia DHFR was located within the chromosome, and was associated with the integrase gene of Tn*7*. The high prevalence of this DHFR located within the chromosome and its association with Tn*7* has been observed previously in a number of studies (Heikkilä *et al* 1991; Chatkaeomorakot *et al* 1987; Towner *et al* 1994). The different size restriction fragments of chromosomal DNA which hybridised to probes for the type Ia DHFR and the integrase gene of Tn*7* in each isolate suggest that the DHFR is not located within a complete Tn*7* transposon, or is integrated into different sites within the chromosome. Both these phenomena have been observed previously (Heikkilä *et al* 1991). Only one isolate was found to harbour this DHFR on a non-transferable plasmid which hybridised to a probe for the integrase gene of Tn*21*. The association of the *dhfrIa* gene cassette with the integrase gene of Tn*21* has been reported previously (Heikkilä *et al* 1991; Sundström and Sköld 1990; Towner *et al* 1994). All the transferable plasmids harbouring the type Ia DHFR exhibited distinct restriction profiles. This suggests that its wide dissemination may be due to transposase or integrase activity. Four of these plasmids conferred resistance to streptomycin, spectinomycin and trimethoprim but not sulphonamides suggesting that the type Ia DHFR may be located on Tn*7* in these isolates. Only one of these plasmids hybridised to the integrase gene of Tn*21*.

Contrary to most previous data, the type Ib DHFR, which occurred in 21.8% of the isolates, was shown to be the most prevalent DHFR in South African isolates of commensal faecal flora. Since the development of the type Ib oligonucleotide probe,

the type Ib DHFR has been shown to occur in several centres around the world at considerable frequency. Young *et al* (1994) found it to be the dominant DHFR on transferable trimethoprim resistance plasmids in Dundee. The same probe has shown that the type Ib has been found in 14% of faecal isolates conferring transferable trimethoprim resistance from South India (Tait and Amyes 1994). Hybridisation between the type Ib DHFR with the whole gene probe for the type V DHFR has been observed (Tait and Amyes 1994; Young *et al* 1994). Because of the high degree of homology shared between the type V and Ib (88%) at the nucleotide level (Young *et al* 1994) this has probably led to the type Ib being overlooked at the expense of the type V DHFR. It appears that the type Ib and V occur concurrently at most centres.

Table 4.2. Frequency of resistant DHFRs in South African commensal faecal flora.

DHFR	Transferred by conjugation (%)	Non-Transferable (%)	Total (%)
Type Ia	11 (3.1)	41 (11.5)	52 (14.6)
Type Ib	58 (16.2)	20 (5.6)	78 (21.8)
Type V	25 (7.0)	3 (0.8)	28 (7.8)
TypeVI	0	0	0
Type VII	5 (1.4)	62 (17.4)	67 (18.8)
Type IIb/c	0	0	0
Type IIIa	0	0	0
Type VIII	45 (12.6)	1 (0.3)	46 (12.9)
Type IX	0	0	0
Type X	0	0	0
Type XII	1 (0.3)	0	1 (0.3)
Type XIII	30 (8.4)	15 (4.2)	45 (12.6)
Unknown	22 (6.2)	23 (6.4)	45 (12.6)
Total	196	161	357

In the South African isolates, the plasmids harbouring the type Ib DHFR transferred by conjugation in 75% (58/78) of the isolates which harboured this DHFR gene. The high incidence of the type Ib DHFR gene on self-transmissible plasmids in South Africa results partly to the presence of one plasmid, pUK2301 which accounted for (26/78) of the type Ib bearing isolates. The large number of different plasmids carrying the type Ib (24) suggest that this gene may have disseminated amongst these plasmids via transposase or integrase activity. The type Ib DHFR gene was initially

isolated from a Tn*7* like transposon Tn*4132*. Downstream of this gene is a 59-base element similar to that of the type V DHFR suggesting it forms part of a cassette-like structure (Young *et al* 1994). In this study, some of the plasmids harbouring the type Ib DHFR including two which could not be mobilised by conjugation, hybridised to a probe for the integrase gene of Tn*21* suggesting that the DHFR may be inserted into different integron structures in a similar manner to *dhfrIa* as reported by Sundström and Sköld (1990). This association of *dhfrIb* with the integrase gene of Tn*21* has been reported previously by Tait and Amyes (1994).

In contrast to the type Ia DHFRs, the DHFR genes, which probed positive for the type Ib DHFR but did not transfer by conjugation, were located on plasmids and not on the chromosome in the isolates that were tested. These plasmids appear to be stable and could not transfer their resistance by conjugation under a variety of conditions. The origins and mechanisms of dispersal of these plasmids is difficult to determine. One possible explanation is in the nature of the plasmids: the plasmids entering the cell by conjugation, may have undergone subsequent modification by a number of potential mechanisms which change the nature of a plasmid thus causing it to lose its ability to transfer to a recipient strain. These include insertion, deletion or rearrangement of the plasmids by various insertion sequences or by transposase or integrase activity (Carlos and Miller 1980). Another possible reason for the failure of these plasmids to transfer by conjugation is the failure of the host bacterium to develop the necessary conjugative structures with the recipient bacterium. It appears that plasmids may account for a larger proportion of non-transferable resistance factors than previously expected.

The type VII DHFR was the second most prevalent DHFR in South Africa. The presence of the type VII DHFR in South Africa is consistent with the findings of other workers. Its prevalence is widespread and has been isolated in Sweden, Finland, Nigeria, Sri Lanka (Sundström *et al* 1993) and in the UK (Amyes *et al* 1989). What is unusual, is that the incidence of the type VII DHFR recorded in this study is considerably higher than that found in any previously studied population. More fascinating is the contrast between the small number, out of the total sample of 357 isolates, bearing this gene on self transmissible plasmids (5/67) in comparison to the large number of isolates that harbour this gene on the chromosome. The number of different species carrying the type VII DHFR gene and the different fragment sizes which hybridised to the type VII DHFR and Tn*21* integrase genes, suggest that the

widespread dissemination of this DHFR gene is not the result of the spread of a single clone but rather the dispersal of this gene on mobile DNA elements.

The type VII DHFR has in previous studies been located within a Tn*21*-like element on transferable plasmids (Sundström *et al* 1993) although no other locations for the type VII gene has been documented in the literature. The close association of the type VII DHFR gene with the integrase gene of Tn*21* in the South African population suggests that the type VII DHFR gene is integrated into an integron-like structure. The number of different sized restriction fragments that showed this combination of these two genes suggest that the integrase may be incorporated into a number of different deletion products or precursor structures of Tn*21*-like transposons (Zühlsdorf and Wiedemann 1992) such as Tn*8056* (Sundström *et al* 1993). Unlike Tn*7*, site-specific insertion of Tn*21* has not been documented which suggests that these elements insert at random sites into the chromosome. It is unclear why the integrase gene of Tn*21* which is normally associated with DHFR genes on plasmids (Sundström *et al* 1988; Sundström *et al* 1993; Heikkilä *et al* 1991; Heikkilä *et al* 1993) should show a preference for integration into the chromosome. Further molecular characterisation of sequences flanking this DHFR and associated integrase should clarify this phenomenon. This also raises questions surrounding the efficiency of transfer of a transposable element from the chromosome of one organism to the chromosome of another via a self-transferable plasmid intermediate.

Despite the recent discovery of the type VII DHFR, this DHFR has been detected in isolates collected from as early as 1974 (Sundström *et al* 1993). There are a number of reasons why this DHFR may have been overlooked: for one, this DHFR exhibits similar biochemical properties to other type I like DHFRs, and from most of the European studies, this enzyme appears to make up only a small percentage of trimethoprim-resistant DHFRs that are detected (Sundström *et al* 1993; Towner *et al* 1991). Furthermore, if the infrequent occurrence of this DHFR on self-transmissible plasmids in South Africa is consistent in other parts of the world it is likely that this DHFR may not be detected or its prevalence underestimated in studies which have only focused on plasmid mediated DHFRs, such as those by Towner *et al* (1991) and Tait and Amyes (1994).

Little is known about the prevalence of the type IIIc/VIII DHFR. This gene was first isolated after an outbreak of *S. sonnei* in North Carolina, USA and was subsequently found to be widespread in *S. sonnei* isolates around the USA (Parg *et al* 1990). This

DHFR gene has also been isolated from urinary isolates in Sweden (Sundström *et al* 1991a). In South African isolates of faecal flora it was shown to be second most prevalent DHFR on transferable plasmids 23% (45/196) and the fourth most prevalent DHFR overall 12.9% (46/357). In complete contrast to the type VII DHFRs, there was a marked difference between the number of isolates that harboured the type VIII DHFR on transferable plasmids (45/46) in comparison to only one isolate that was unable to transfer this DHFR to a recipient strain. The presence of a number of geographically ubiquitous plasmids harbouring the type VIII DHFR in South African isolates suggests that these are the principal means of dispersal of the type VIII DHFR in South Africa. The type VIII DHFR is one of the few trimethoprim-resistant DHFRs which have not been found as gene cassettes associated within an integron.

The type VIII DHFRs accounted for all the transconjugants bearing intermediate MICs of less than 1024mg/l though it did include some isolates with higher MICs. Two different MICs for trimethoprim have been previously reported for this DHFR, 256mg/l (Barg *et al* 1990) and 1000mg/l (Sundström *et al* 1991a). The range of MICs reported in this study are thought to result from differences in expression of the DHFR by different plasmids and differences in plasmid copy number. With the exception of one *S. sonnei* isolate, all the other isolates were from *E. coli*. The American and most of the South African isolates carried resistance to trimethoprim, ampicillin, streptomycin, and sulphamethoxazole (Barg *et al* 1990). It is not known whether there is any common genetic link between these isolates. No other prospective study has looked for this particular DHFR. Its ubiquitous presence in South Africa and the USA suggest that it is a prevalent DHFR amongst faecal organisms.

The type V DHFR is geographically widespread and has been isolated from most of the studies of resistant DHFR genes at low-to-moderate frequency (Table 1.4); however it should be kept in mind that some of these enzymes may have been misidentified type Ib DHFRs. In South Africa, the prevalence of this DHFR 7.8% (28/357), is similar to that reported in other parts of the world. As shown in table 4.2, 89% (25/28) of the isolates harboured this DHFR on transferable plasmids. The remaining three isolates harboured this DHFR on non-transferable plasmids. The location of this DHFR on plasmids within the integron of Tn*21* has been reported previously (Sundström *et al* 1988; Tait and Amyes 1994). In the South African isolates, 60% (15/25) of the transferable plasmids and all the non-transferable plasmids hybridised to the probe for the integrase gene of Tn*21*.

In South Africa, the type XII DHFR was shown to be rare, and was isolated from only one isolate. This newly discovered enzyme has been shown to occur at relatively low frequency at a number of centres. It has previously been isolated in Finland from a urinary isolate and from four *Shigellas* of Asian origin (Heikkilä *et al* 1993). It has also been detected in a number of isolates from children attending day-care centres in Texas (Singh *et al* 1992b). The high level of homology between the nucleotide sequence of the types XII and XIII DHFRs (85%) is similar to that shared between the types Ib and V DHFR genes (88%) which suggests that, like the type Ib and V DHFRs, a whole gene probe from either the type XII or XIII will cross-hybridise between the two genes.

The novel type XIII DHFR was fairly prevalent in South Africa and occurred in 12.6% (45/357) of the isolates. Two-thirds of the isolates which hybridised to the oligonucleotide probe for the type XIII DHFR could transfer the DHFR by conjugation. The integrase gene of Tn*21* was associated with the type XIII DHFR in 40% (12/30) of the plasmids. It appears that, like the type XII DHFR, the type XIII DHFR can be located with or without the presence of the integrase gene of Tn*21* (Heikkilä *et al* 1993; Singh *et al* 1992b). Although only one plasmid harbouring this DHFR gene was detected in more than one isolate, a number of restriction profiles of plasmids harbouring this DHFR were similar and shared a number of identical sized restriction fragments. This suggests that the plasmids which harboured this DHFR gene have undergone a significant amount of rearrangement and divergent evolution.

The successful spread of this DHFR may be due to the extremely high incidence of resistance associated with this DHFR gene. Considering that resistance to ampicillin, tetracycline, sulphonamides, streptomycin, spectinomycin and chloramphenicol occurred in more than half of the plasmids, which harbour the type XIII DHFR, it is likely that antimicrobial therapy with any of these agents will exert strong selection pressure for bacteria harbouring these plasmids. It is difficult to predict how widely distributed the type XIII DHFR may be in other parts of the world. However, since this DHFR was extremely widespread in South Africa, and was detected in all the rural and urban population groups, it is thought that this DHFR may be widely distributed in other parts of the world and may account for a proportion of the 10 to 68% of DHFR genes which were not identified in a number of studies (Heikkilä *et al* 1990b; Jansson *et al* 1991; Singh *et al* 1992b; Chang *et al* 1992; Towner *et al* 1994).

Probes for the type IIb and IIc DHFRs did not hybridise to any DHFRs from the South African isolates. Despite a number of studies which showed a high prevalence of type II DHFRs, the type II DHFRs were absent, or extremely rare in most of the studies (Table 1.4). The high incidence of these DHFRs recorded in some of the studies are most likely due to a number of factors. The large size of the gene probes used in these studies have subsequently been shown to contain part of a Tn*21*-like transposon, and as a result are likely to hybridise with any of the DHFRs Ia, Ib, II, V, VII, X, XII and XIII which have been found associated with the integrase gene of Tn*21*. Furthermore, control strains from the more recently detected DHFRs (Ib, IIIb, IIIc/VIII, IV, V, VI, VII, IX, X, XII and XIII) were not available as adequate controls for these early type II DHFR probes. As shown in figure 3.3. even with the relatively small 280-bp type II DHFR gene probe, the stringency of the washes during hybridisation are critical to preventing cross-hybridisation.

Despite the widespread use and availability of a gene probe for the type IIIa DHFR, this DHFR has only been detected in one *Salmonella typhimurium* isolate from New Zealand (Fling *et al* 1988) and at varying frequency in isolates from Nottingham, UK (Towner *et al* 1994). The absence of the type IIIa DHFR from a number of studies (Table 1.4) including South Africa suggest that this DHFR is fairly rare.

There are no epidemiological data for the prevalence of the type VI DHFR. Despite the fact that this DHFR was first detected in a urinary isolate of *Proteus mirabilis* from South Africa (Wylie *et al* 1988), it was not detected in any of the faecal isolates from this study. A study by Sundström *et al* (1993) failed to detect type VI in a collection of isolates from a number of countries which suggests that this DHFR is a rare enzyme. There is no epidemiological data for the prevalence of the type X DHFR. The failure to detect this DHFR in South Africa suggests that its distribution may be localised. The absence of the type IX DHFR in South African populations and others (Jansson *et al* 1992) suggest that the type IX DHFR is restricted to isolates from swine and some human isolates in Sweden.

Of the 45 isolates which did not hybridise to any of the DHFR probes, half of these isolates (22/45) transferred trimethoprim resistance to a recipient strain, suggesting that in these isolates resistance was conferred by the production of an additional resistant DHFR. The MIC for trimethoprim in all the transconjugants was ≥2048mg/l. The high MICs of these unidentified DHFRs suggests that resistance was unlikely to be conferred by either the type IIIb or type IV DHFR. Although the type II DHFRs

are known to confer high levels of resistance, the possibility of them being either a type IIa or type II variant, which did not hybridise to the type IIb DHFR probe, are remote, since not only are these DHFRs rare and appear to be restricted to clinical isolates, but these DHFRs are also usually associated with the integrase gene of Tn*21*; an association which did not occur in these isolates. These unknown enzymes are most likely to be novel DHFRs, possibly a type I-like enzyme, or a variant of the highly resistant type XII and XIII DHFRs. The large number of different restriction profiles and antibiogram types representing the unknown DHFRs (Table 4.3) suggest that there may be more than one novel DHFR gene amongst these isolates.

Table 4.3. Plasmids conferring unknown trimethoprim-resistant DHFRs, their resistance profile, and their association with In21.

Plasmid	Isolates	In21	Antibiogram
pUK2326	1	-	Tp
pUK2322	1	-	TpSx
pUK2342	1	-	TpSx
pUK2363	1	-	TpSxSp
pUK2341	1	-	TpSmSp
pUK2317	6	-	TpTcSxSp
pUK2335	1	-	TpTcGmSx
pUK2369	1	-	TpTcApSxSp
pUK2370	2	-	TpTcApSxSp
pUK2403	1	-	TpTcApStSp
pUK2383	1	-	TpTcApSxStCm
pUK2397	1	-	TpTcApGmSxSmStSpCm
pUK2398	1	-	TpTcApGmTnCazSxSmStSpCm
pUK2399	2	-	TpTcApGmTnCazCtxSxSmStSpCm

A number of mechanisms of resistance to trimethoprim have been considered in the 23 isolates which did not transfer trimethoprim resistance. Considering that (22/23) of these isolates conferred trimethoprim MICs of ≥2048mg/l, it is most likely that resistance is due to the production of either a plasmid or transposon mediated DHFR. One of these isolates was a *Pseudomonas aeruginosa* which was most likely to be intrinsically resistant to trimethoprim (Grey and Hamilton-Miller 1977). All of these isolates grew on DM minimal medium and therefore cannot be thymine mutants. It is also unlikely that these isolates are resistant as a result of membrane impermeability or

mutations to the chromosomal DHFRs since these mechanisms of resistance usually confer low to intermediate trimethoprim MICs (Gutmann *et al* 1985; Grey *et al* 1979). Only one isolate (*Klebsiella* spp.) was resistant to low levels of trimethoprim (MIC=16mg/l).

When the amino acid sequence of the type XIII DHFR was compared to the sequences of the other trimethoprim-resistant DHFRs, the highest degree of homology shared with this DHFR was the type XII (82.4%) (Table 4.4) which suggests that this DHFR is closely related to and may have arisen from the same ancestor as the type XII. With the exception of the type XII, the type XIII DHFR shared a limited amount of homology (22.4% to 34.5%) with the other resistant DHFRs (Table 4.4). A similar level of homology (15.2% to 35.5%) occurred between the type XIII DHFR and the DHFR genes of other species (Table 4.5). The most closely related of these DHFRs to the type XIII DHFR was the chromosomal DHFR of *Bacillus subtilis* (35.8%) and the DHFR genes of *Enterobacteriaceae* (34.5-35.2%).

Table 4.4. Amino acid sequence identity of the type XIII DHFR with trimethoprim-resistant DHFRs isolated from bacteria.

Trimethoprim-resistant DHFR (Bacterial)	Identity with type XIII DHFR (%)
Type Ia	27.9
Type Ib	33.9
Type V	33.9
Type VI	29.1
Type VII	34.5
Type IIIa	30.3
Type IIIc/VIII	32.7
Type IV	22.4
Type IX	25.5
Type X	22.4
Type XII	82.4
Staphylococcus aureus S1	30.3

From the limited number of DHFRs from different species that have been sequenced, there are no enzymes that are closely related enough to the type XIII DHFR to make an accurate assessment to determine the putative origins of this DHFR. However,

since the type XIII DHFR is of a similar length and shares the closest homology with bacterial DHFRs, the most likely ancestor of the type XIII DHFR is thought to be bacterial. The presence of the type XIII DHFR and a number of other resistant DHFRs as gene cassettes suggests that the promoter sequences of the original host of the DHFR are not recognised in Gram-negative bacteria. Inserting such gene cassettes into the promoters of integrons and other DNA elements, which are recognised in Gram-negative bacteria, may be necessary for gene expression in these organisms.

Table 4.5. Amino acid sequence identity of the type XIII DHFR with other chromosomal DHFRs.

Chromosomal DHFRs	Identity with type XIII DHFR (%)
Bacterial	
Bacillus subtilis	35.8
Citrobacter freundii	35.2
Enterobacter aerogenes	35.2
Klebsiella aerogenes	35.2
E. coli	34.5
Neisseria gonorrhoeae	33.9
Halobacterium volcanii	30.9
Lactobacillus casei	29.6
Staphylococcus aureus	26.1
Enterococus faecium	26.0
Other	
Drosophila melanogaster	29.1
Pneumocystis carinii	26.1
Candida albicans	22.4
Leishmania amazonensis	21.8
Leishmania major	20.6
Chicken	20.6
Mouse & Hamster	20.0
Bovine & Porcine	19.4
Saccharomyces cerevisiae	19.4
Plasmodium falciparum	19.4
Herpes saimiri (virus)	18.2
Plasmodium chabaudi	17.6
Human	15.2

The type XIII DHFR exhibited unique biochemical properties and, with the exception of the type II DHFRs, is the most resistant DHFR to inhibition by trimethoprim that has been characterised. The trimethoprim ID_{50} for the type XIII DHFR (800μm) was considerably lower than the atypical type II enzymes (ID_{50}=20 000-80 000μM) but when compared to the other resistant DHFRs, with the exception of the type VI (ID_{50}=200μM), the ID_{50} was more than 10 times higher than the resistant type I-like enzymes (ID_{50}=23-57μM). The K_i values which are a better indicator of the performance of a DHFR, in the presence of trimethoprim, showed that the type XIII DHFR (K_i=180μM) was resistant to a similar order of magnitude as the type IIs (K_i =150-6100μM) and was also more resistant than any other of the resistant DHFRs including the type VI (K_i=75μM) which has often been described as having type II like properties (Amyes *et al* 1992b).

What separates the type XIII DHFR more dramatically from the type II enzymes is the sensitivity of the type XIII DHFRs and other DHFR types to methotrexate. The methotrexate ID_{50} value for the type XIII is 5μM in comparison to 750-1100μM for the type IIs. The relative sensitivity of the type XIII DHFR to methotrexate confirms that the active site is structurally homologous to that of the *E. coli* chromosomal DHFR (Matthews *et al* 1986). Heat-sensitivity is another factor which separates the type XIII DHFR (TD_{50}=7 min) from most other DHFRs because, with the exception of the type I-like enzymes which are very unstable (TD_{50}=0.5-1.5 min), most other DHFRs such as the type IIs, IIIa, IIIb, IV, S1 and the chromosomal DHFRs show no loss of activity after 12 min exposure at 45°C. Only the type VIII DHFR (TD_{50}=8 min) shows a similar heat-sensitivity profile to the type XIII.

Overall, the most similar of the resistant DHFRs to the type XIII, in terms of their biochemical properties, were the type I-like enzymes, a relationship which is reflected to some extent by the level of similarity between the amino acid sequence of these genes (Figure 4.1). Although amino acid identity was less spectacular, many of the different amino acids in positions 1-80 were conservative replacements.

Most of the biochemical properties of the type XII DHFR have not yet been determined. The only available data are a trimethoprim ID_{50} value of 700μM, which is almost identical to the type XIII DHFR. Structurally, the type XII and XIII DHFRs are similar and, with the exception of one conservative amino acid replacement, all the amino acids at the trimethoprim, methotrexate and NADPH cofactor binding sites are

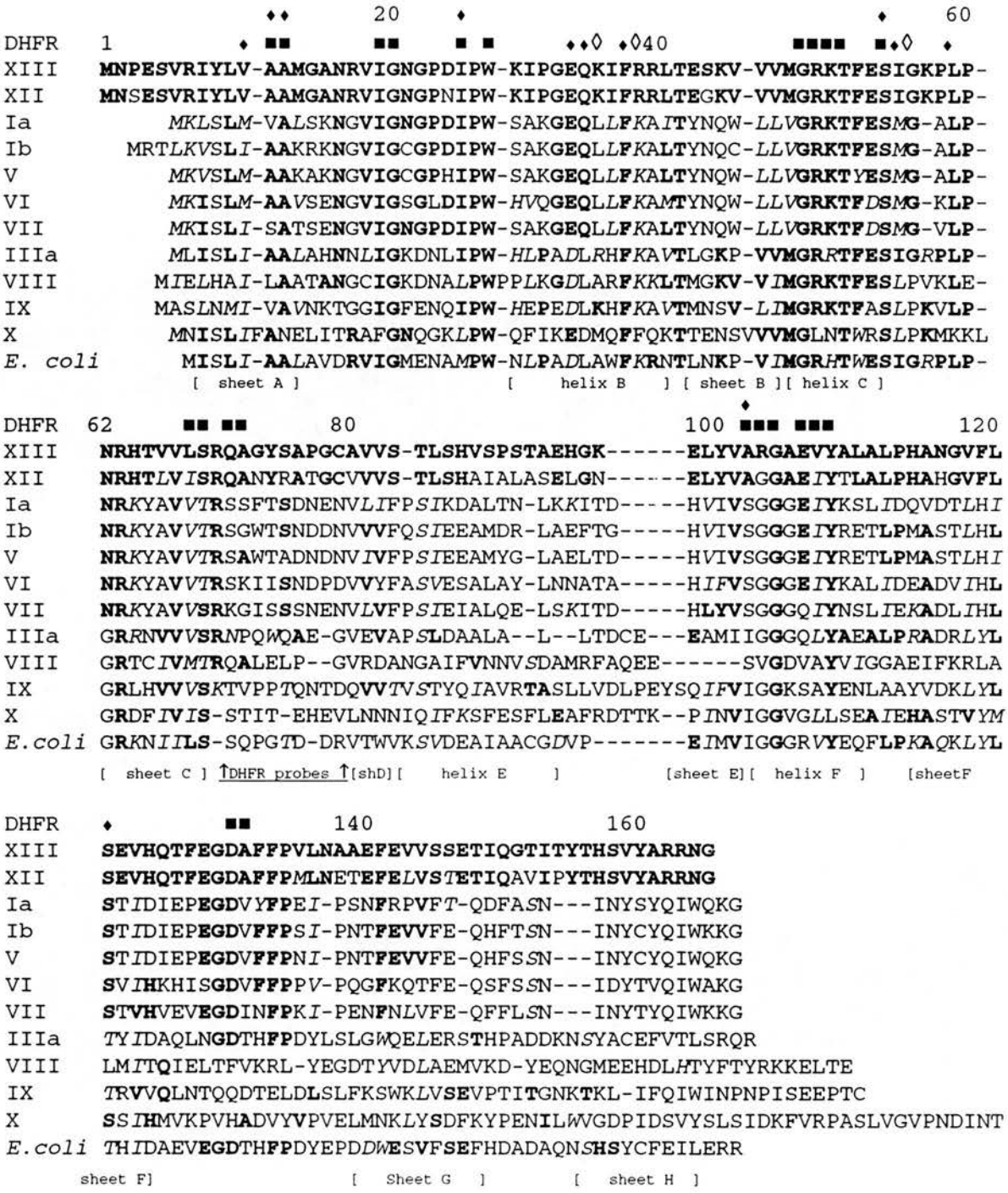


Figure 4.1. Amino acid sequence similarities between the type XIII, XII, Ia, Ib, V, VI, VII, IIIa, VIII, IX, X and the chromosomal DHFR from *E. coli* K12. Positions containing identical amino acids with respect to the type XIII DHFR are in bold script, and conserved replacements within the groups (D,E), (F,W,Y), (H,K,R), (I,L,M,N), (N,Q) and (S,T) are in italics. Positions involved in binding trimethoprim (♦), methotrexate (♦ and ◊) and NADPH cofactor (■) based on the *E. coli* K12 DHFR (Rouch *et al* 1989) are indicated.

identical (Rouch *et al* 1989). The six consecutive amino acids from positions 88-94 of the type XIII DHFR (Figure 4.1) are dissimilar to those of the type XII DHFR. The first three amino acid substitutions are caused by a frame shift which occurs over six nucleotides as a result of a base deletion at position 263 and a subsequent base insertion at position 269 on the nucleotide sequence of the type XIII DHFR gene (Figure 3.19). This substituted region suggests that the type XIII DHFR has diverged from the type XII or a type XII-like ancestor, since the same region in the type XII shares close homology to other DHFR types including the chromosomal DHFR of *E. coli*. Furthermore, the amino acids at positions 80 and 151 of the type XIII DHFR are dissimilar to the type XII and other resistant DHFR types which confirm this evolutionary divergence. This pattern is not entirely consistent as the amino acids that differ at positions 24 and 75 of the type XII and XIII DHFR are more conserved in the type XIII DHFR.

Only half the trimethoprim binding sites on the trimethoprim sensitive chromosomal DHFR of *E. coli* (as indicated by Rouch *et al* 1989) (Figure 4.1) were conserved in the type XIII DHFR. Six of the amino acids, that are involved in binding trimethoprim at positions 12, 13, 36, 54, 55 and 59, were identical. The binding sites at positions 11, 25, 32, and 121 were conservative substitutions, and the trimethoprim binding sites at positions 33 and 102 were different. The significance of these alterations in the trimethoprim binding sites in the type XIII is difficult to predict, without knowing the three dimensional structure of the enzyme as determined by X-ray crystallography. However, by comparing the differences between the trimethoprim binding sites of the sensitive *E. coli* chromosomal enzyme, with those of a trimethoprim-resistant vertebrate DHFR (chicken), for which a three dimensional structure is available for both enzymes, a model for the differences in selectivity can be established.

The following model for selectivity has been described by Baccanari and Kuyper (1993). In the *E. coli* chromosomal DHFR structure, trimethoprim forms five hydrogen bonds with the enzyme, including an ionic interaction between the pyrimidine ring of trimethoprim and the carboxylic acid side-chain of aspartic acid (position 27) (Figure 4.2). This enzyme-ligand interaction cannot occur with the substrate dihydrofolate and is, in part, responsible for the enhanced binding of trimethoprim to the enzyme. The conformation of trimethoprim is different in vertebrate enzymes such that when the ionic bond forms with the carboxylic acid sidechain of glutamic acid (position 30) which is equivalent to aspartic acid at position 27 in the *E. coli* DHFR (Figure 4.2), the trimethoxyphenyl group is positioned in an

alternative binding pocket (away from the cofactor) and cannot establish ligand-cofactor interactions. The binding mode also alters the orientation of the pyrimidine portion of trimethoprim. The pyrimidine is pushed further into the cleft such that its 4-amino group cannot form a hydrogen bond with valine (position 115), the vertebrate enzyme amino acid residue analogous to isoleucine (position 94) of the *E. coli* DHFR. Thus, a combination of the altered conformation and hydrogen bonding pattern of trimethoprim may in part, be responsible for species differences in affinity.

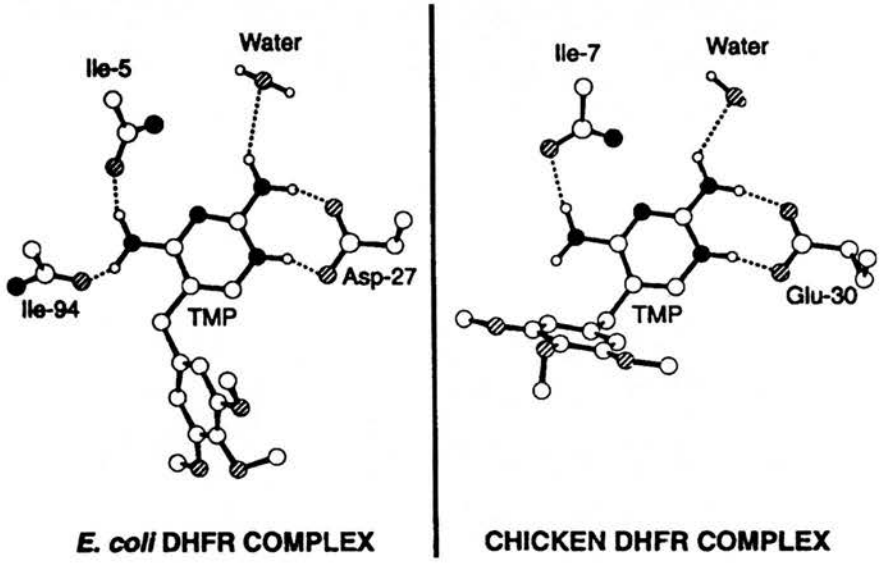


Figure 4.2. Comparison of the conformation and hydrogen bonding pattern of trimethoprim in the *E. coli* and chicken DHFR ternary complexes (Figure from Baccanari and Kuyper 1993).

Trimethoprim binding to the resistant type XIII DHFR is fairly analogous to that of the vertebrate enzyme. The most significant similarity is the substitution of a glutamic acid residue at position 32 of the type XIII DHFR for the aspartic acid residue which corresponds to position 27 of the sensitive *E. coli* chromosomal DHFR. An identical substitution also occurs in the corresponding position of the vertebrate, type I-like and type XII DHFRs (Sundström *et al* 1993; Heikkilä *et al* 1993). The amino acid substitution at position 33 from leucine to glutamine of the type XIII DHFR may enhance a change in orientation of the trimethoprim molecule when it binds to the glutamic acid residue of the DHFR. This substitution is also present in the type I-like and type XII DHFRs. Another significant change is the substitution of alanine for isoleucine at position 102 of the type XIII (position 94 in *E. coli*). Since isoleucine at

position 94 of the *E. coli* enzyme forms a hydrogen bond with the 2-amino group, a substitution of a dissimilar amino acid at the corresponding position may prevent this.

The K_m of the type XIII DHFR is approximately ten times higher than that of the chromosomal DHFR of *E. coli*, and is one of the highest recorded for trimethoprim-resistant DHFRs. This suggests that the changes within the active site which affect trimethoprim binding have resulted in a reduction of the affinity of the substrate for the type XIII DHFR. Despite the low turnover rate of tetrahydrofolate by the type XIII DHFR, the high K_i of the type XIII DHFR is sufficient to confer significant MICs of trimethoprim (>2048mg/l).

The elements of secondary structure of the chromosomal DHFR of *E. coli* were determined by X-ray crystallographic analysis and it was shown that DHFR enzymes consist of a central eight stranded β-sheet, and four α-helices where the remaining residues make up loops connecting these elements of secondary structure as reviewed by Baccanari and Kuyper (1993). These features of secondary structure appear to be similar between the different DHFRs and are marked according to the amino acid sequence of *E. coli* (Figure 4.1). The secondary structure of the type XIII DHFR differs from that of the *E. coli* chromosomal DHFR in that the loop structure between β-sheet C and β-sheet D of the type XIII DHFR contains two amino acid insertions, the start of α-helix E is shortened by one amino acid and the loop between α-helix E and β-sheet E has an extra amino acid inserted.

One of the interesting features of the alignment of the amino acid sequence of the trimethoprim-resistant DHFRs with the features of secondary structure, is that, with the exception of the first 60 amino acids which contain most of the conserved trimethoprim and NADPH cofactor binding sites, the most heterogeneous regions are the loop structures that occur between the β-sheet and α-helix structures. Evolutionary divergence at these loops as a result of genetic drift can be expected since changes in amino acid structure in these regions are unlikely to affect DHFR function. These regions are therefore perfect for the development of oligonucleotide probes for distinguishing between resistant DHFR genes, because not only is the heterogeneity clear between DHFR genes which have already been sequenced, but also heterogeneity in undiscovered genes is more likely to be greater in these regions than others.

There are three loops which occur in DHFR genes which are ten or more amino acids long and are therefore suitable for the construction of DHFR probes (Figure 4.1). They are located between β-sheet C and β-sheet D, between α-helix E and β-sheet E and between β-sheet F and β-sheet G. The loop between β-sheet C and β-sheet D is the most promising since not only does the length of this loop vary between the more distantly related DHFRs, but also the nucleotide sequence of the most closely related DHFRs such as the types Ib and V and the types XII and XIII are the most heterogeneous in this region. It was from this region of the gene that the oligonucleotide probes for the DHFR genes used in this study were derived (Figure 4.3). The loop between α-helix E and β-sheet E is the most variable between the more distantly related groups, which vary in loop length by as much as seven amino acids. Unfortunately, in this region both the types XII and XIII and the family I DHFRs are too conserved. The loop between β-sheet F and β-sheet G appears to be conserved even between some of the more distantly related DHFRs such as the type IIIa, XIII, the family I DHFRs and the chromosomal DHFR of *E. Coli*.

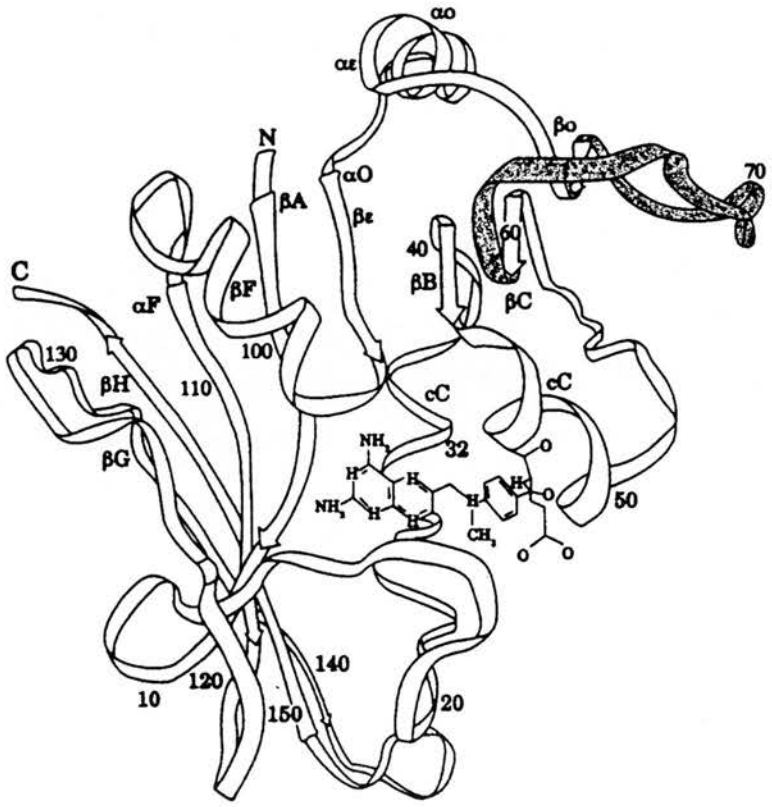


Figure 4.3. The three-dimensional structure of the *E. coli* DHFR showing the attachment sites of methotrexate (Williams 1995). The shaded region indicates the loop between β-sheet C and β-sheet D used in the design of oligonucleotide probes.

CONCLUSIONS

The commensal faecal flora of healthy individuals from South Africa form a large and important reservoir of trimethoprim-resistant dihydrofolate reductase genes. This presents a threat to the continued use of trimethoprim in South Africa, including a number of antimicrobial agents which are linked to trimethoprim resistance.

The amino acid loops which link the elements of secondary structure of DHFR genes are highly heterogeneous and are ideal regions for the development of discriminating oligonucleotide probes for the detection of trimethoprim-resistant DHFRs. Oligonucleotide probes developed from the nucleotide sequence of the loop between β-sheet C and β-sheet D were developed, and were shown to distinguish between closely related DHFR genes, and did not hybridise with any of the DHFR related sequences. The highly selective DHFR oligonucleotide probes were able to distinguish between the closely related type XII and XIII DHFR genes and resulted in the detection of the latter.

The epidemiology of resistant DHFR genes in South Africa is distinctly different from the data from Europe where the type Ia is the predominant DHFR gene. The more recently detected DHFR genes such as the type Ib, VII, VIII and XIII were shown to be highly prevalent in South Africa, and, from the recent data collected from other parts of the world, suggest that these DHFRs are also prevalent in other parts of the world.

The molecular epidemiology and genetic location of the DHFR genes differed remarkably between the different DHFR types. The distribution of the DHFR genes which could not transfer their resistance by conjugation is completely different from the distribution of these genes located on transferable plasmids. The prevalence of the type Ib, V and VIII DHFR, which occurred predominantly on transferable and non-transferable plasmids, was a complete contrast to the types Ia and VII DHFRs which were located predominantly on the chromosome. This suggests that the genetic structures associated with these genes such as Tn*21* and Tn*7* play an important role in the dissemination and spread of these genes.

It is important that the levels of antibiotic resistance in South Africa are carefully monitored as multiple resistant organisms continue to limit the choice of antimicrobial agents available for therapy.

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